Aquatic Toxicology 212 (2019) 54–69

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Physiological trade-offs, acid-base balance and ion-osmoregulatory plasticity in European sea bass (*Dicentrarchus labrax*) juveniles under complex scenarios of salinity variation, ocean acidification and high ammonia challenge

Jyotsna Shrivastava*, Moses Ndugwa, Warren Caneos, Gudrun De Boeck

Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp, Belgium

ARTICLE INFO

Keywords:
Global change
Marine teleosts
Rhesus (Rh) glycoproteins
Osmotic stress
Ammonia excretion rate
Ion-regulation
Respiratory acidosis

ABSTRACT

In this era of global climate change, ocean acidification is becoming a serious threat to the marine ecosystem. Despite this, it remains almost unknown how fish will respond to the co-occurrence of ocean acidification with other conventional environmental perturbations typically salinity fluctuation and high ammonia threat. Therefore, the present work evaluated the interactive effects of elevated pCO_2, salinity reduction and high environmental ammonia (HEA) on the ecophysiological performance of European sea bass (*Dicentrarchus labrax*). Fish were progressively acclimated to seawater (32 ppt), to brackish water (10 ppt) and to hyposaline water (2.5 ppt). Following acclimation to different salinities for at least two weeks, fish were exposed to CO_2-induced water acidification representing present-day (control pCO_2, 400 µatm, $LoCO_2$) and future (high pCO_2, 1000 µatm, $HiCO_2$) sea-surface CO_2 level for 3, 7 and 21 days. At the end of each exposure period, fish were challenged with HEA for 6 h (1.18 mM representing 50% of 96 h LC_{50}). Results show that, in response to the individual $HiCO_2$ exposure, fish within each salinity compensated for blood acidosis. Fish subjected to $HiCO_2$ were able to maintain ammonia excretion rate (J_{amm}) within control levels, suggesting that $HiCO_2$ exposure alone had no impact on J_{amm} at any of the salinities. For 32 and 10 ppt fish, up-regulated expression of Na^+/K^+-ATPase was evident in all exposure groups (HEA, $HiCO_2$ and HEA/$HiCO_2$ co-exposed), whereas $Na^+/K^+/2Cl^-$ co-transporter was up-regulated mainly in $HiCO_2$ group. Plasma glucose and lactate content were augmented in all exposure conditions for all salinity regimes. During HEA and HEA/$HiCO_2$, J_{amm} was inhibited at different time points for all salinities, which resulted in a significant build-up of ammonia in plasma and muscle. Branchial expressions of Rhesus glycoproteins (Rhcg isoforms and Rhbg) were upregulated in response to $HiCO_2$ as well as HEA at 10 ppt, with a more moderate response in 32 ppt groups. Overall, our findings denote that the adverse effect of single exposures of ocean acidification or HEA is exacerbated when present together, and suggests that fish are more vulnerable to these environmental threats at low salinities.

1. Introduction

Anthropogenic factors leading to emission of carbon dioxide (CO_2) such as fossil fuel combustion and land use changes (excessive deforestation) are rapidly raising the atmospheric CO_2 concentration level at a rate that is unprecedented in the last 22,000 years (IPCC, 2013; Joos and Spahni, 2008). It is projected that by 2100, the atmospheric CO_2 concentration will increase to 541–970 ppm (IPCC, 2014). As much of this anthropogenically generated CO_2 is absorbed by the oceans, the increase of atmospheric CO_2 is leading to an enormous elevation in the average partial pressure of CO_2 (pCO_2) in the ocean, resulting in a fundamental shift on the ocean's carbonate chemistry besides the rise in the temperature. Carbon dioxide will dissolve in seawater forming carbonic acid (H_2CO_3), therefore, an increment of pCO_2 is eventually leading to a gradual decline in the ocean pH in a process known as ocean acidification (OA). In the last 200 years, the global ocean pH has dropped by 0.1 pH units (i.e. from 8.2 to 8.1, 30% increase in acidity). Recent projection suggests that by early in the next century, oceanic pCO_2 could reach 1000 µatm, reducing surface water pH by 0.3–0.5 units (170% increase in acidity), which is more than 100 times as rapid as at any time over the past hundreds of the millennia (Allmon and Esbaugh, 2017; Clements and Chopin, 2016; Dore et al., 2009; Meure

* Corresponding author.
E-mail address: jyotsna.ua@gmail.com (J. Shrivastava).

https://doi.org/10.1016/j.aquatox.2019.04.024
Received 10 April 2019; Received in revised form 30 April 2019; Accepted 30 April 2019
Available online 02 May 2019
0166-445X/

et al., 2006).

Ocean acidification will impact marine organisms across all levels of biological organization, from cellular to the ecosystem levels. However, the most direct effect of ocean acidification on fish is altered blood CO_2 chemistry, leading to narcotizing acidosis (Esbaugh et al., 2012, 2016; Green and Jutfelt, 2014; Heuer et al., 2016; Strobel et al., 2012). Blood acidosis could lead to a reduction in Hb–O_2 binding affinity via the Root–Bohr effect (Claiborne, 1998; Wedemeyer, 1997). It is well established that over 90% of total CO_2 in the blood is present as plasma HCO_3^-. As such, the respiratory acidosis compensation in marine fish is principally achieved by adjusting plasma HCO_3^- levels through the differential regulation of HCO_3^- and H^+– effluxes, which are widely believed to be coupled to the influx of Na^+ and Cl^-. H^+ is believed to be transported across the gill apical membrane primarily through the Na^+/ H^+ exchangers (NHE) while HCO_3^- is transported back into the plasma by the electrogenic basolateral Na^+/ HCO_3^- co-transporter (NBC). NBC can elevate plasma HCO_3^- level, thereby offsetting the increase in plasma CO_2 and returning pH to resting levels. However, it has not been fully confirmed in marine teleost gill ionocytes whether additional bicarbonate can be gained by active HCO_3^-/Cl^- ion exchange (Tresguerres and Hamilton, 2017). Though CO_2 excretion is a diffusion limited transport system, it has also been shown recently that Rhesus (Rh) glycoproteins can act as putative CO_2 channel proteins (Esbaugh et al., 2016; Perry et al., 2010) indicating that CO_2 and ammonia transport might interact.

Recent investigation have shown that ocean acidification can induce numerous challenges in fish, including disturbance in acid-base and osmotic homeostasis, respiratory dysfunction, interference with ion exchange, predator avoidance, altered aerobic performance, olfaction and vision impairment, metabolism depression and enzyme inhibition (Heuer and Grosell, 2014), which can negatively affect overall fitness and survival (Baumann et al., 2012; Miller et al., 2012). These changes in the ocean chemistry will threaten not only the global wild fish stock, but are also expected to have dramatic impacts on the sustainability of fish and shellfish aquaculture (Ellis et al., 2017; FAO, 2014). In this era of global change it is likely that the effect of OA may not act in isolation, rather it may interact with other environmental or anthropogenic generated anomalies threatening the integrity of marine ecosystems. The effects of OA have become a well-studied topic over the past decades. However, a gap exists in the literature on the ability of fish to respond to the concurrent occurrence of ocean acidification with other environmental stressors.

Estuaries in particularly are susceptible for OA, owing to the additional inputs of acidifying factors including urban run-off, microbial respiration, and eutrophication (Cai et al., 2011; Esbaugh et al., 2016; Feely et al., 2010). Consequently, it is believed that due to the interaction with anthropogenic environmental processes, estuarine environments are likely to experience enhanced acidification as a result of overall increased CO_2 production (Esbaugh et al., 2016; Feely et al., 2010; Melzner et al., 2013; Wallace et al., 2014). Together with elevated CO_2, estuarine ecosystem continuously experience large fluctuation in salinity. The salinity gradient of estuaries, coastal lagoons, and the inshore water has gradually reduced over last few decades (IPCC, 2013). Ongoing global warming and its effect on melting of glaciers and ice caps as well as frequent intense rainfall are the major causes resulting in the decline of the salinity gradient of these natural ecosystems. Euryhaline teleosts including diadromous and non-diadromous fish often inhabit estuaries at different stages of their life cycle as part of migration and spawning, and therefore are frequently challenged with salinity mediated osmotic stresses. Lowered ambient salinity also results in major changes in seawater chemistry, and modifies responses to OA in estuarine organisms. Similar to OA, salinity stress can strongly affect iono- osmoregulation and acid–base homeostasis (Kelly and Woo, 1999; Roessig et al., 2004; Sinha et al., 2015a; Yan et al., 2004).

In coastal waters, OA and osmotic challenge events can be accompanied by high environmental ammonia (HEA). HEA results from the anthropogenic inputs of nitrogen, excretion of cultured animals (cages and pens), mineralization of organic detritus, sewage effluents, industrial wastes and agricultural run-off. Similar to OA and salinity anomalies, HEA can also induce a number of ecotoxicological effect on physiological processes including gas exchange, acid–base balance and ionic/osmotic regulation. As mentioned above, interactions between HEA and OA are not unlikely as the ammonia transporting Rh glycoproteins are also suspected to be putative CO_2 channel proteins (Esbaugh et al., 2016; Perry et al., 2010). As such it would be of importance to understand how fish will potentially respond to multiple stressors such as OA, salinity reduction and ammonia when occurring concomitantly.

Fish gills are the primary site for gas exchange, ion regulation, acid–base balance and nitrogenous waste excretion. Since gills are the prime tissue to be exposed to the variations of the external milieu, it is likely that responses attributable to single stressers/pollutants can be different from those mediated by an assortment of multiple stressors. A high plasticity of the gill ion-transporting and ammonia excreting ability is necessary to cope with a wide range of environmental salinities, OA as well as HEA events. The ion transport mechanisms in marine teleosts gills are coordinated by ion channels, cotransporters (e.g., $Na^+/K^+/2Cl^-$ (NKCC), Na^+/Cl^- (NCC)) and energy dependent ion transport enzymes like the Na^+/K^+-ATPase (NKA) (Hiroi and McCormick, 2012; Hwang et al., 2011). NKA is present on the basolateral membrane of gill epithelium that actively transports Na^+ out and K^+ into the cells, and also play a key role in generating a driving force for many ion-transporting systems in the gills (Hirose et al., 2003; Hwang and Lee, 2007; Lin et al., 2004a, b; Marshall and Bryson, 1998). Basolateral localized NKCC is reported to play a vital role in the maintenance of the electrolyte concentration, and transepithelial ion and water movement in polarised cells (Cutler and Cramb, 2001; Russell, 2000). In addition, ion regulation in fish gills is closely associated with ammonia excretion pathways (Wilkie, 1997, 2002). Similarities in the hydration radius of K^+ and NH_4^+ can allow substitution at transport sites, therefore, NKA and NKCC are believed to be involved in ammonia excretion (Alam and Frankel, 2006; Randall et al., 1999; Wilkie, 1997). In addition, the key involvement of Rh glycoproteins in diffusive NH_3 efflux has been documented in fish (Nakada et al., 2007; Nawata et al., 2007). Of particular relevance as putative CO_2 channel, these Rh glycoproteins also function as ammonia channels, binding NH_4^+ but facilitating the diffusion of NH_3 (Nawata et al., 2010).

To date, the majority of the research has focused on the impact of a single stressor; however, the combined effects of OA, salinity reduction and HEA on the physiological fitness of fish are not well understood and require further investigation. Furthermore, in order to evaluate the individual and interactive effects these three environmental stressers, estuarine fish can potentially act as ecologically and environmentally relevant models. As an estuarine-dependent fish species, European sea bass (*Dicentrarchus labrax*) is the preferred test organism for this study. This is a commercially important fish species for fisheries and aquaculture. Global capture and culture production of European sea bass totaled 162,172 tonnes, with a worth over $US 1 billion (FAO, 2012). Therefore the potential effects of near-future oceanic conditions either along or together with salinity challenge and HEA on European sea bass could have clear ecological and economic implications.

With this background, the current study aims to assess the combined effect of multiple-stressors encompassing increasing environmental pCO_2 (400–1000 µatm), reduced seawater salinities (32 ppt- 2.5 ppt) and ammonia exposure on coping strategies in European sea bass. We hypothesize that elevated pCO_2 combined with salinity stress as well as HEA would interact to affect blood chemistry, acid–base status, ammonia homeostasis and physiological stress response. In brief, to achieve our goals we determined (i) blood pH, plasma pCO_2 and HCO_3^- level as prime indices of acid-base balance; (ii) ammonia metabolism and excretion dynamics by quantifying ammonia excretion rate and its accumulation in plasma and muscle; (iii) expression patterns of

Table 1
Water chemistry parameters of the experimental conditions.

Parameter	Exposure conditions					
Salinity	32 ppt		10 ppt		2.5 ppt	
pCO_2 (μatm)	400	1000	400	1000	400	1000
pH	8.2	7.7	7.8	7.4	7.4	7.0
T (°C)	17 ± 1	17 ± 1	17 ± 1	17 ± 1	17 ± 1	17 ± 1
TA (μmol/kg)	3094.6 ± 63.2	2874 ± 57.6	1132.3 ± 13.4	1081.8 ± 12.9	362.6 ± 37.4	334.6 ± 37
DIC (μmol/kg)	2755 ± 56.2	2753.8 ± 55.6	1106.9 ± 14.6	1106.4 ± 13.8	376 ± 38	375.5 ± 38.4
HCO_3^-(μmol/kg)	2472.7 ± 43.2	2603.2 ± 48.2	1060.9 ± 11.9	1055.5 ± 12.3	356.5 ± 36.3	332.6 ± 36.6
CO_3^{2-}(μmol/kg)	270.4 ± 9.4	119.8 ± 4.4	31.9 ± 0.7	12.6 ± 0.3	3.2 ± 0.6	1.1 ± 0.2
$[CO_2]$ (μmol/kg)	11.9 ± 0.01	30.8 ± 0.04	14.1 ± 0.01	38.3 ± 0.03	16.2 ± 0.1	41.8 ± 0.1

Data are presented as means ± S.D. pCO_2 represents the partial pressure of CO_2, T (°C): water temperature, TA: the total alkalinity, DIC: total dissolved inorganic carbon, CO_3^{2-} : carbonates, HCO_3- : bicarbonate and $[CO_2]$ were calculated using CO_2 SYS software.

branchial Rh glycoproteins to understand whether or not Rh proteins play a dual role in facilitating- ammonia as well as CO_2 transport; (iv) the ion-regulatory status by investigating the branchial NKA and NKCC transcript levels; and (v) glucose and lactate levels in plasma as indicators of stress response.

2. Materials and methods

2.1. Experimental design

The effects of three factors were assessed in this study: salinity, pCO_2 and high environmental ammonia (HEA). Experiments were carried out at three salinity levels, 32 ppt (normal seawater), 10 ppt (brackish water) and 2.5 ppt (hyposaline water) and two pCO_2 levels of 400 μatm and 1000 μatm, yielding six experimental groups. The two selected pCO_2 levels are representative of the present-day conditions (400 μatm, $LoCO_2$) and pCO_2 level projected by the IPCC for the next century (1000 μatm, $HiCO_2$). The group subjected to a salinity of 32 ppt and a pCO_2 of 400 μatm was considered as the control group. In order to explore the combined effect of the salinity and ocean acidification (elevated pCO_2) on the ammonia tolerance of the fish, each of these six experimental groups were subsequently challenged with HEA at 1.18 mM which represents 50% of 96 h LC_{50} (50% of the concentration that is lethal to 50% of the population in 96 h; Person-Le Ruyet et al., 1995).

2.2. Experimental animals and maintenance

European sea bass (*D. labrax*) juveniles (32.5 ± 1.9 g; 12–16 months old) were obtained from Ecloserie Marine (Gravelines, France) and transferred to the Department of Biology, University of Antwerp. Fish were maintained in three 600 L re-circulatory tanks, filled with artificial seawater (Marinemix® Professional salt, 32 ppt). Thereafter, a total of 360 fish were distributed into eighteen 200 L re-circulatory tanks (n = 20 per tank; 32 ppt) placed in a climate chamber where temperature was adjusted at 17 ± 1 °C and photoperiod was 12 h light and 12 h dark. Fish were acclimated to the above mentioned constant salinity, temperature and photoperiod for one month prior to the experiment, and were fed with commercial pellets (Skretting, Boxmeer, The Netherlands) at a rate of 2% on their wet body weight/day. Water quality (in both 600 L and 200 L tanks) was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. All animal experiments were approved by the local ethics committee, University of Antwerp (EDC permit 2014-23), and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations.

2.3. Experimental set-up

2.3.1. Fish pre-acclimation to lowered seawater salinities

Following the acclimation period, fish in twelve tanks were progressively acclimated to two hypoosmotic experimental salinities: 10 ppt (˜249 mOsm/kg, pH 7.8; 6 tanks) and 2.5 ppt (˜69 mOsm/kg, pH 7.4; 6 tanks), whereas fish in the remaining six tanks were maintained at normal seawater salinity 32 ppt

(˜800 mOsm/kg, pH 8.2). Changes in salinity were progressed by reducing the salinity by 5% each three days until 10 ppt was reached. Thereafter, salinity was slowly reduced (˜2% every 5 days) until salinity of 2.5 ppt was achieved. Experimental salinities were adjusted by diluting artificial seawater with filtered freshwater, and salinity was measured using a hand-held refractometer. Each experimental group was acclimatized to the desired salinity for at least 2 weeks and was fed daily at a rate of 2% of their wet body weight.

2.3.2. CO_2 exposure

After being acclimatized at the respective salinities for 2 weeks, pCO_2 level in three tanks (out of six tanks) belonging to each of the salinity regimes was increased until the desired pCO_2 levels of 1000 μatm was reached, while in remaining three tanks (for each salinity groups) ambient pCO_2 level (˜ 400 μatm) was maintained. pCO_2 was induced via a computerized feedback system which equated to the ambient pH and pH at the elevated pCO_2 level. To determine the pH level corresponding to pCO_2 levels at three salinity regimes (32, 10 and 2.5 ppt), total dissolved Inorganic Carbon (DIC) and total alkalinity (TA) was quantified at the start of the experiments and at each water change using triplicate samples from the experimental tanks. These measurements were then entered into a CO_2 system calculation program (CO_2SYS software, Macro Version 2.2; Pierrot et al., 2006) to calculate the desired pH values corresponding with the pCO_2 levels (Table 1). DIC was determined using a Shimadzu TOC SSM-5000 A module (Shimadzu Europa GmbH, Germany). Phosphoric acid was added to the sample and the resulting CO_2 was purged at 200 °C and measured.

Pure compressed carbon dioxide gas was pumped into the tanks from an external CO_2 cylinder (Praxair NV, Schoten Belgium). In essence, pCO_2 level was obtained with the aid of an automatic system (Cap CTRL software) and a negative feedback controller/trigger box (DAQs, Loligo Systems) which controlled the pCO_2 level in each tank by adjusting desired pH set points. The pH electrodes (3310, WTW, Germany) were calibrated with tris (tris hydroxymethyl aminomethane) buffers (GE Healthcare Bio-Sciences AB, Uppsala Sweden), with the same salinity and ionic strength as the experimental tanks. Tris buffers thus served as the pH standard for calibration and for the daily monitoring of the pH set points. CO_2 dissolves rapidly in the seawater, carbonate chemistry equilibrates less quickly (as a function of temperature) and thus an overshoot (hysteresis) of pH was generated, thus

in the present experiment, the pH set values were maintained within hysteresis ± 0.07 units. The experimental set up consisted of fish subjected to pCO_2 and salinity treatments for a period of 3, 7 and 21 days.

2.3.3. High environmental ammonia (HEA) exposure

During each time period (3, 7 and 21 days) a total of 8 fish (three fish from the first two tanks and two fish from the third tank) were randomly collected from salinity and pCO_2 experimentation tanks and transferred to their respective 200 L ammonia exposure tanks (water volume set to 160 L) with the salinity and pCO_2 matching the experimentation tanks within the same climate chamber. Fish were placed in the tanks the evening before the ammonia exposure started and left overnight to settle with continuous aeration. Feeding was suspended 24 h before the fish transfer in ammonia exposure tanks, which provided sufficient time for the gut to be emptied and to stabilize the endogenous fraction of nitrogenous waste excretion. The details of exposure regimes and sampling schedule are illustrated in Supplementary file 1.

The experimental protocols consisted of exposing the fish with 1.18 mM HEA (representing 50% of 96 h LC_{50} value for ammonia) for 6 h. The exposure tank was spiked with the required amount of an NH_4HCO_3 stock solution (Sigma, Germany). A constant concentration of 1.17 ± 0.09 mM of (total) ammonia was maintained throughout the experiment. Ammonia concentrations were measured using the salicylate–hypochlorite method (Verdouw et al., 1978). After 6 h of HEA exposure, each of the 8 fish was transferred individually into their respective flux chambers. The containers used were sealable Nalgene containers of 5 L (water volume set to 1.5 L). The experimental containers were shielded with black plastic to minimize the visual disturbance. Water composition was identical to that of the ammonia exposure tanks and fitted with individual air-stones. For parallel control (no HEA), a set of another 8 fish (three fish from the first two tanks and two fish from the third tank) from the experimental tanks were transferred directly into respective flux boxes. For the ammonia excretion measurements, initial water samples (duplicate 2 mL) were taken followed by a final water sample collection after 2 h for both control and HEA groups.

Ammonia excretion rates J_{amm} (μmol/g/h) were calculated as:

$$J_{amm} = ([Amm]_i - [Amm]_f) \times V/(t \times M)$$

where $[Amm]_i$ and $[Amm]_f$ are the initial and final concentrations of ammonia in the water (in μmoles/L) obtained from comparison to a standard curve. V indicates volume (L), t time (h), and M mass (g).

2.4. Sampling procedure and analysis

Following the final water samplings, the fish were terminally anaesthetized with a lethal dose of MS-222 (ethyl 3-aminobenzoate methane-sulphonic acid, 1 g/L, Acros Organics, Geel, Belgium) neutralized to the relevant pH, blotted dry and weighed. Subsequently, a blood sample was collected from the caudal blood vessel using a heparinised syringe. Blood pH was measured immediately with a pH probe (744 pH Meter, Metrohm, Switzerland). Thereafter, blood was centrifuged immediately (for 1 min at 16,000 rpm at 4 °C) and plasma total carbon dioxide (TCO_2) concentrations were measured by a carbon dioxide analyzer (Corning 965, Midland, MI, USA). Plasma pCO_2 was calculated from TCO_2 and pH, using the solubility of carbon dioxide (α_{CO2}) and the apparent pK (pK_{app}) according to Boutilier et al. (1984). Plasma samples were then frozen in liquid nitrogen and stored at −80 °C for later analysis of metabolites i.e. ammonia, lactate and glucose content. Subsequently, fish were dissected on ice, gills and muscle were collected, snap frozen in liquid nitrogen and stored at −80 °C for later molecular analysis and muscle ammonia content assay respectively.

2.5. Analytical techniques

2.5.1. Plasma metabolites

Plasma ammonia, lactate and glucose concentration were determined using commercial enzymatic kits (R-Biopharm AG, Darmstadt, Germany).

2.5.2. Muscle ammonia concentration

Muscle ammonia was determined according to Wright et al. (1995). Frozen muscles samples (0.5–1 g weight) were ground to fine powder in liquid nitrogen, deproteinized in 1 vol of ice-cold perchloric acid (8.4%) and centrifuged for 10 min (4 °C). The supernatant was removed, neutralized with saturated $KHCO_3$ and centrifuged again. The final supernatant was then analyzed for ammonia levels using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

2.6. Molecular analyses

Total RNA was isolated from gill samples using Trizol (Invitrogen Merelbeke, Belgium) according to the manufacturer's instructions. The extracted RNA samples were DNase treated to avoid genomic DNA contamination. DNase buffer (DNase I buffer with $MgCl_2$; Fermentas, UK), DNase I (Fermentas, UK) and RNase inhibitor (Fermentas, UK) were added to the isolated RNA followed by the incubation at 37 °C for 30 min. Phenol–chloroform extraction and washing steps with various dilutions of ethanol were then performed to remove any traces of DNA or salts. The quantity of the RNA was evaluated by using Nano- Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). The purity was checked by measuring the OD260/OD280 absorption ratio (> 1.95).

2.6.1. Real-time PCR

For quantitative real-time PCR (qPCR), a starting amount of 1 μg RNA was transcribed into first-strand cDNA using the Revert Aid H minus First Strand cDNA Synthesis Kit (Fermentas, Cambridge, UK). mRNA expression of Na^+/K^+-ATPase alpha subunit 1 (α-subunit1), Rhcg1, Rhcg2, Rhbg and $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) in the gills was quantified by qPCR using the specific primers listed in Table 2. qPCR analyses were performed on an Mx3000 P QPCR System (Agilent Technologies, Belgium). Reactions containing 5 μL of 5 × diluted cDNA, 10 pmol each of forward and reverse primers, 0.3 μL ROX dye (1:500 dilution) and 10 μL Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent) were performed in a four-step experimental run protocol: a denaturation program (3 min at 95 °C); an amplification and quantification program repeated 40 times (15 s at 95 °C, 20 s at 60 °C); a melting curve program (60–95 °C with a heating rate of 0.10 °C/s and a continuous fluorescence measurement) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, 'no-template' controls (i.e. with water sample) for each set of genes were also run to ensure no contamination of reagents, no primer–dimer formation, etc.

Comparison of two reference genes (elongation factor-1α and18S rRNA) favored elongation factor-1α (EF-1α) as the most stable gene across the samples (20 random samples were tested) and was used as endogenous standard to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

2.7. Statistical analysis

All data have been presented as mean values ± standard error (S.E.). The normality of the data was checked using the Shapiro-Wilk test. For comparisons between different experimental groups a one-way analysis of variance (ANOVA) was performed followed by the least significant difference (LSD) test. Student's two-tailed *t*-test was used for single comparisons. Main effects of salinity challenge, ocean

Table 2
qPCR primer sequences, accession numbers and calculated efficiency.

Gene	Primer sequence (5′→3′)	Efficiency	References	Accession no.
Na^+/K^+-ATPase (α-subunit1)	Forward: CTGGAGTGGAAGAAGGTC Reverse: GATGAAGAGGAGGAAGG	109.8%	Giffard-Mena et al., 2008	AY532637
$Na^+/K^+/2Cl^-$ cotransporter 1	Forward: TCATCACTGCTGGAATCTT Reverse: AGAGAAACCCACATGTTGTA	113.4%	Lorin-Nebel et al., 2006	AY954108
Rhbg	Forward: CCTCATGGTGACCCGAATCC Reverse: GCCTGCACTCTGTCCACATA	101.7%	Blondeau-Bidet et al. (2019)	
Rhcg1	Forward: TCAGGGAATTGTGTGACCGC Reverse: AGAATCAAGTCCACGCTGGG	97.0 %	Blondeau-Bidet et al. (2019)	
Rhcg2	Forward: TGGCTACCTGTTTGTCACGC Reverse: GGATGCTCGGCGGCTTTATA	105.1%	Blondeau-Bidet et al. (2019)	
EF 1α	Forward: GCTTCGAGGAAATCACCAG Reverse: CAACCTTCCATCCCTTGAAC	97.5%	Geay et al., 2010	AJ866727
18S rRNA	Forward: CGCTAGAGGTGAAATTCTTGGA Reverse: GATCAGATACCGTCGTAGTTCC	107.8%	Hakim et al., 2009	AM419038

The accession number refers to the registered sequence from GenBank.

acidification, ammonia exposure and time, and their interactions were analyzed by four-way ANOVA. The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 20.0. A probability level of 0.05 was used for rejection of the null hypothesis. Principal component analysis (PCA) was performed by using OriginLab 9 software (OriginLab, Northampton, MA, U.S.A.). All measured parameters were subjected to PCA to investigate the overall effect of exposure treatments on integrated compensatory responses. The standardized scores of the first two components which explained the highest variation were used to make biplots.

3. Results

3.1. Ammonia excretion rate

The effect of OA as an individual factor on the ammonia excretion rate (J_{amm}) was observed only for 2.5 ppt acclimated fish, wherein exposure to $HiCO_2$ for 3 days induced an increase ($P < 0.05$) in J_{amm} relative to the control (400 µatm) (Fig. 1). J_{amm} in both $LoCO_2$ and $HiCO_2$ groups were invariably declined (or even reversed) during HEA exposure in all the tested salinity regimes. In 32 ppt, HEA exposure resulted in the reversal of the ammonia flux for both $LoCO_2$ and $HiCO_2$ fish at day 21. Likewise, for the 10 ppt acclimated fish, J_{amm} was reversed in HEA- $HiCO_2$ as well as HEA- $LoCO_2$ groups at day 3 and day 7 relative to the naïve (non-HEA) group. At 2.5 ppt, a reduction in J_{amm} for the HEA- $HiCO_2$ co-exposed group was noted at 7 and 21 days whereas a similar decline for the HEA- $LoCO_2$ group was observed at day 3. Comparison among $LoCO_2$ groups of seawater (32 ppt), brackish water (10 ppt) and hyposaline water (2.5 ppt) acclimated fish shows that J_{amm} tended to be lower, which was significant during day 3 at 10 and 2.5 ppt compared to the respective seawater -acclimated fish and in some other groups at various time points. Overall, a significant interaction was observed between pCO_2, salinity challenge, HEA, and time on J_{amm} (Table 3).

3.2. Blood and plasma chemistry

3.2.1. Blood pH

pCO_2 itself had no effect on blood pH level under the experimental conditions, except for a significant reduction in 10 ppt salinity following 21 days of exposure to $HiCO_2$ (Fig. 2). Effect of HEA was noted for both pCO_2 levels at all tested salinities, wherein HEA exposed fish (at day 7 in 32 ppt and 2.5 ppt, day 21 in 10 ppt) had elevated ($P < 0.05$) blood pH relative to non-HEA naïve counterparts. Overall, blood pH was notably lower in all experimental groups acclimated to 2.5 ppt compared to 32 ppt and 10 ppt counterparts.

Plasma total CO_2 (TCO_2) was measured and plasma pCO_2 and

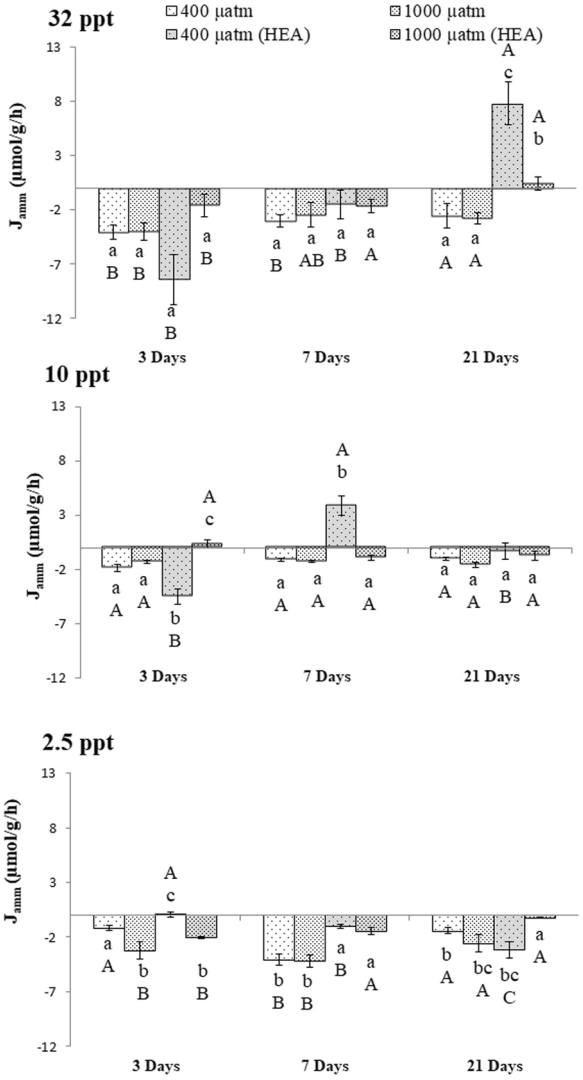

Fig. 1. Ammonia excretion rate (µmol/g/h) in fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–c) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

Table 3
The effects of pCO_2 levels, different salinity regimes, ammonia exposure and time, and their interactions on the physiological parameters in European sea bass.

	Value	J_{amm}	Blood pH	Plasma T_{amm}	Muscle T_{amm}	Glucose	Lactate	NKA	NKCC1	Rhcg1	Rhcg2	Rhbg
Salinity	*F*	9.66	59.03	68.45	69.42	10.40	20.04	15.54	23.86	5.69	9.11	5.00
	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01
pCO_2	*F*	0.20	6.86	3.24	28.64	0.11	19.18	34.36	2.91	9.29	0.07	0.00
	P	0.65	0.01	0.08	0.00	0.74	0.00	0.00	0.9	0.00	0.80	0.96
HEA	*F*	5.88	6.10	9.53	1.36	1.38	12.17	5.91	5.58	3.90	0.03	0.36
	P	0.02	0.02	0.00	0.25	0.24	0.00	0.02	0.02	0.05	0.87	0.55
Time	*F*	7.7	1.27	33.00	28.58	8.12	4.79	18.25	11.82	0.95	8.62	13.23
	P	0.00	0.28	0.00	0.00	0.00	0.01	0.00	0.00	0.39	0.00	0.00
Salinity x pCO_2	*F*	0.19	2.12	13.57	12.45	2.49	14.70	0.94	0.70	2.47	0.78	13.23
	P	0.82	0.12	0.00	0.00	0.09	0.00	0.40	0.50	0.31	0.46	0.00
Salinity x HEA	*F*	2.28	1.20	3.53	7.24	0.63	2.47	4.97	8.12	1.17	0.74	2.04
	P	0.11	0.30	0.04	0.00	0.534	0.09	0.01	0.00	0.32	0.48	1.38
Salinity x Time	*F*	14.85	8.68	22.00	6.47	13.30	6.82	2.18	6.38	14.01	1.22	4.92
	P	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.310	0.00
pCO_2 x HEA	*F*	0.59	2.70	0.01	1.14	6.22	7.89	4.06	0.97	2.97	0.14	0.62
	P	0.44	0.10	0.94	0.29	0.02	0.01	0.05	0.33	0.09	0.70	0.44
pCO_2 x Time	*F*	10.54	2.98	12.78	5.01	2.73	23.04	9.99	0.20	2.72	0.93	4.32
	P	0.00	0.06	0.00	0.01	0.07	0.00	0.00	0.82	0.07	0.34	0.02
HEA x Time	*F*	15.78	0.03	0.40	20.23	3.17	2.26	3.53	2.64	1.98	0.64	11.60
	P	0.00	0.97	0.67	0.00	0.05	0.11	0.04	0.08	0.15	0.53	0.00
Salinity x pCO_2 x HEA	*F*	1.24	1.53	1.33	19.01	3.52	0.08	11.30	1.84	3.43	0.87	7.52
	P	0.29	0.20	0.27	0.00	0.04	0.93	0.00	0.17	0.04	0.42	0.00
Salinity x pCO_2 x Time	*F*	10.77	2.33	12.04	22.05	5.00	7.63	21.38	9.02	22.82	5.21	11.59
	P	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Salinity x HEA x Time	*F*	7.00	2.97	0.29	3.41	2.37	0.99	7.20	3.27	2.33	0.87	5.77
	P	0.00	0.06	0.89	0.01	0.06	0.41	0.00	0.02	0.06	0.49	0.00
pCO_2 x HEA x Time	*F*	5.25	2.97	0.64	9.19	0.63	9.47	4.94	0.48	1.03	0.50	5.05
	P	0.01	0.06	0.53	0.00	0.53	0.00	0.1	0.62	0.36	0.60	0.01
Salinity x pCO_2 x HEA x Time	*F*	8.37	2.12	0.93	6.16	0.07	1.92	4.34	2.18	0.90	1.22	2.39
	P	0.00	0.08	0.34	0.00	0.98	0.16	0.00	0.10	0.47	0.30	0.06

HCO_3^- calculated but due to the sampling method (caudal sampling), results were highly variable (Supplementary files 2, 3, 4). There were however some trends: at 32 ppt plasma pCO_2 and [HCO_3^-] tended to be higher during $HiCO_2$ exposure, while at lower salinities the opposite occurred and values were lower during CO_2 exposure. There was no clear effect of HEA at any of the salinities.

3.2.2. Plasma ammonia, glucose and lactate

At normal seawater salinity (32 ppt), HEA resulted in a significant increment in plasma total ammonia (T_{amm}) for both $LoCO_2$ and $HiCO_2$ groups at day 7 in comparison to the respective naïve groups (Fig. 3). The same pattern was observed for 10 ppt and 2.5 ppt-acclimated fish at day 3 and day 21 respectively. The increments in T_{amm} in 32 ppt and 10 ppt were followed by a subsequent recovery respectively at day 21 day and day 7–21 to a value not significantly different from their corresponding naïve groups. In addition, following HEA exposure in the 32 ppt group, fish confronted with $HiCO_2$ for 7 and 21 days had higher ($P < 0.05$) plasma T_{amm} relative to HEA exposed- $LoCO_2$ fish. Comparative analysis based on salinities revealed a remarkable lower ($P < 0.05$) T_{amm} content in almost all groups at 2.5 ppt, relative to those at 32 ppt and 10 ppt.

Glucose levels in response to elevated pCO_2 as well as HEA were significantly higher at day 21 for the fish at 32 ppt and 10 ppt (Fig. 4). The same response was noted sooner for 2.5 ppt -acclimated fish, with increments ($P < 0.05$) from day 3 onwards.

For all salinities, ammonia exposure induced plasma lactate accumulation in $LoCO_2$ and $HiCO_2$ treated fish (Fig. 5). At normal seawater, increments in HEA exposed groups were significant at day 3, and at 10 ppt such elevation continued from day 3 until the last day. Fish at 2.5 ppt revealed the similar trend as 10 ppt except at the day 7 when plasma lactate in HEA exposed fish groups was not statistically different from respective naïve fish. In addition, the effect of pCO_2 was noted only at 2.5 ppt; at day 3 and day 21 lactate levels in plasma of $HiCO_2$ fish was considerably elevated ($P < 0.05$) compared to $LoCO_2$ fish.

3.3. Muscle ammonia

At 32 ppt, HEA- $LoCO_2$ fish had higher ($P < 0.05$) muscle ammonia levels relative to their correspondent naïve group at 3 days and 7 days (Fig. 6). For 10 ppt, a similar effect ($P < 0.05$) was noted after 3 days. At lower salinities of 10 ppt and 2.5 ppt, HEA-$HiCO_2$ co-exposed group tended to have higher ammonia content in contrast to its correspondent $HiCO_2$- naïve group. For 10 ppt, the significant different was noted after 3 days and 7 days, while at 2.5 ppt it was apparent after 3 days and 21 days. In contrast to plasma ammonia, fish acclimated to hyposaline water (2.5 ppt) had a higher ammonia content in muscle relative to their counterparts at 32 ppt and 10 ppt (Figs. 3,6).

3.4. Gene expression of NKA

Significant effects of pCO_2, HEA and salinity were seen on NKA mRNA expression levels in gills of experimental fish species (Fig. 7). In 32 ppt and 10 ppt acclimated fish, HEA induced an augmentation in transcript levels at both pCO_2 levels. In 32 ppt, a significant up-regulation was noted from 7 days onwards while at 10 ppt such elevation ($P < 0.05$) was seen from 3 days onwards. Exposure to $HiCO_2$ alone also incited an increase ($P < 0.05$) in expression level, and was seen in 32 ppt (at 21 days) and 10 ppt (at 3 days and 21 days). However, in the hyposaline environment (2.5 ppt), NKA expression level was significantly downregulated in both -$HiCO_2$ (at day 3 and day 21) as well as HEA exposed fish (at day 21). Comparison among salinity regimes revealed a higher expression at 2.5 ppt acclimation relative to those reared at 32 ppt and 10 ppt. Overall, the effect of HEA ($P < 0.05$), salinity challenge ($P < 0.001$), pCO_2 level ($P < 0.001$) and exposure time ($P < 0.001$) in individual as well as in combined exposures ($P < 0.01$) in transcript level of NKA mRNA were significant.

3.5. Gene expression of NKCC1

Branchial NKCC1 mRNA levels in all salinities increased at different

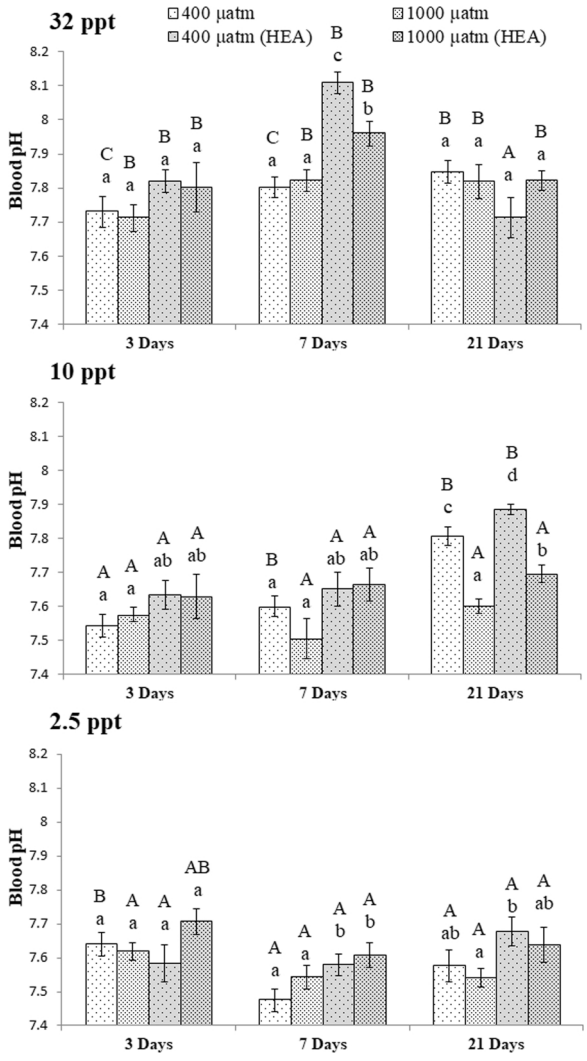

Fig. 2. Blood pH values in fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–d) denotes significant difference between different treatments within same time points. Capital letters (A,B) denotes significant difference between same treatments at different salinities.

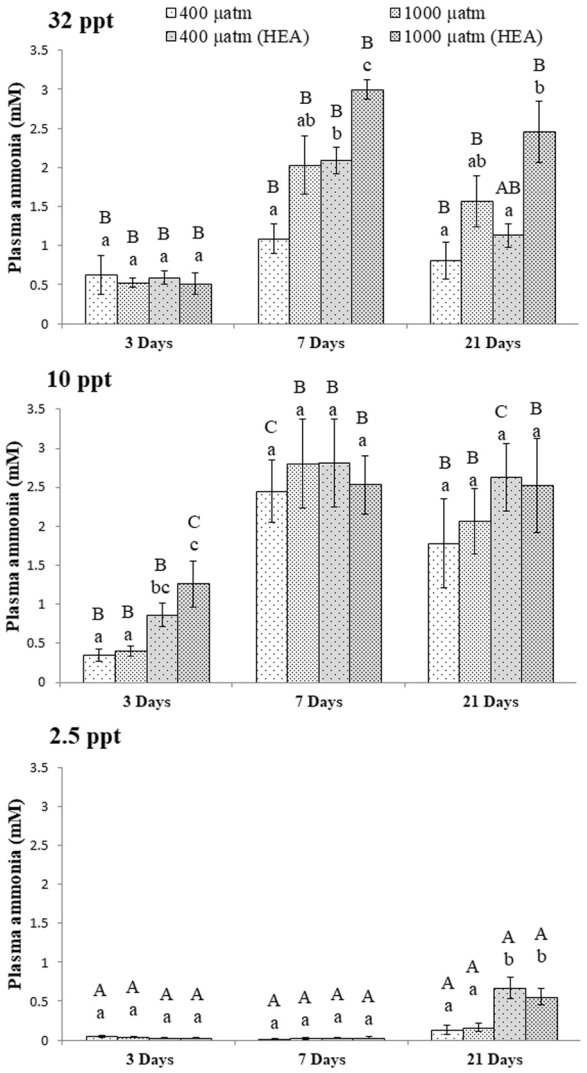

Fig. 3. Total ammonia accumulation (mM) in plasma of fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–c) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

time points when subjected to $HiCO_2$ (Fig. 8). In 32 ppt acclimated fish confronted with $HiCO_2$ for 21 days, mRNA transcript level increased ($P < 0.05$) compared to $LoCO_2$ fish. At 10 ppt and 2.5 ppt a similar trend was noted, the increment was recorded from 7 days onwards. However, no prominent effect ($P > 0.05$) of HEA alone was observed at any tested salinity, except for a momentary increment ($P < 0.05$) at day 3 for 10 ppt-HEA exposed- $LoCO_2$ fish. At 32 ppt (day 21) and reduced salinities (10 ppt and 2.5 ppt, from day 7 onwards), NKCC1 expression level was also significantly up-regulated among $HiCO_2$-HEA co-exposed fish in comparison to the $LoCO_2$ -HEA exposed fish. In addition, relative to 32 ppt the mRNA level in $LoCO_2$ groups at lower salinities reduced considerably ($P < 0.05$).

3.6. Gene expression profile of Rh glycoproteins

$HiCO_2$ exposure significantly increased Rhcg1 expression (relative to control pCO_2) in 32 ppt and 10 ppt salinity groups at 21 days and at 3–21 days respectively (Fig. 9). Conversely, in 2.5 ppt, high CO_2 exposure tended to down-regulate Rhcg1 mRNA level, and a significant reduction was seen at the last experimental period. Similarly, under HEA exposure, an increment at day 21 and day 3 was noted for 32 ppt and 10 ppt respectively, while a reduction was seen at day 21 for 2.5 ppt. Pronounced increments ($P < 0.05$) in Rhcg1 mRNA expression were evident when fish were held at the lowest salinity (2.5 ppt), expression levels increased many folds relative to 32 ppt and 10 ppt counterparts. Overall, a significant interaction was observed between HEA, salinity challenge, and elevated CO_2 on branchial mRNA expression levels of Rhcg1 (Table 3).

Similar to Rhcg1, $HiCO_2$ exposure induced a significant augmentation of Rhcg2 mRNA level at 10 ppt salinity (at 3 days and 21 days), but Rhcg2 expression rate markedly declined ($P < 0.05$) at 2.5 ppt (at 3 day and 21 days) (Fig. 10). A similar trend was seen for Rhbg expression levels (Fig. 11). No prominent effect of HEA was observed in the expression of the Rhcg2 gene, except for an increment ($P < 0.05$) in the $LoCO_2$ group at day 3 at 10 ppt, and a reduction ($P < 0.05$) at day 21 at 2.5 ppt (Fig. 10). HEA exposure induced an increment in Rhbg expression in $LoCO_2$ fish in both 32 ppt (day 7) and 10 ppt (day 3) salinities (Fig. 11). However, at 2.5 ppt a reduction (at day 21) was noted when $HiCO_2$ fish were subjected to HEA (Fig. 11). Analogous to the Rhcg1 expression profile, branchial Rhcg2 mRNA levels were elevated in the fish acclimated at 2.5 ppt in comparison to 32 ppt and 20

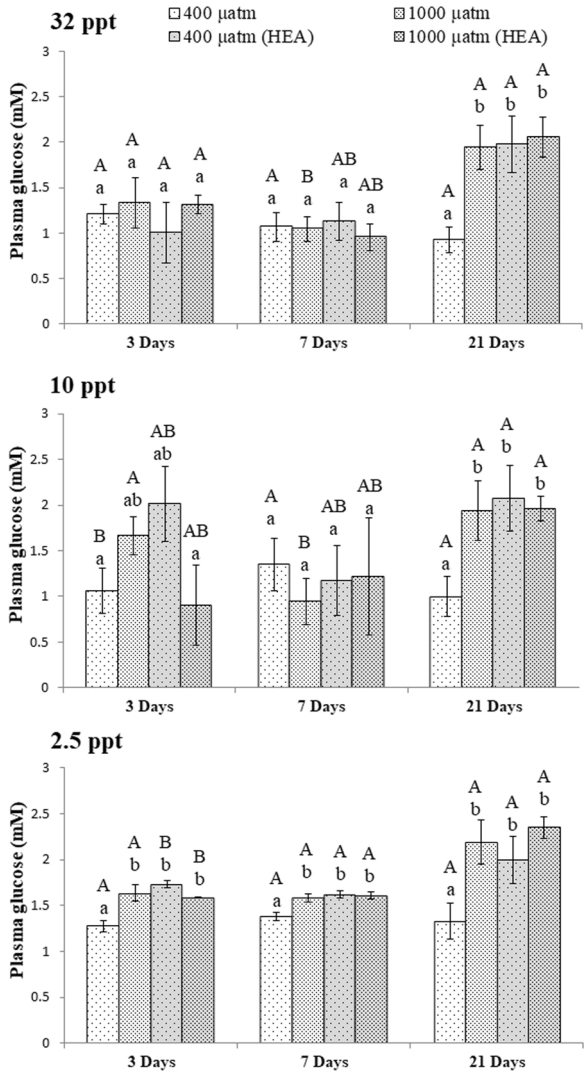

Fig. 4. Glucose level (mM) in plasma of fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a,b) denotes significant difference between different treatments within same time points. Capital letters (A,B) denotes significant difference between same treatments at different salinities.

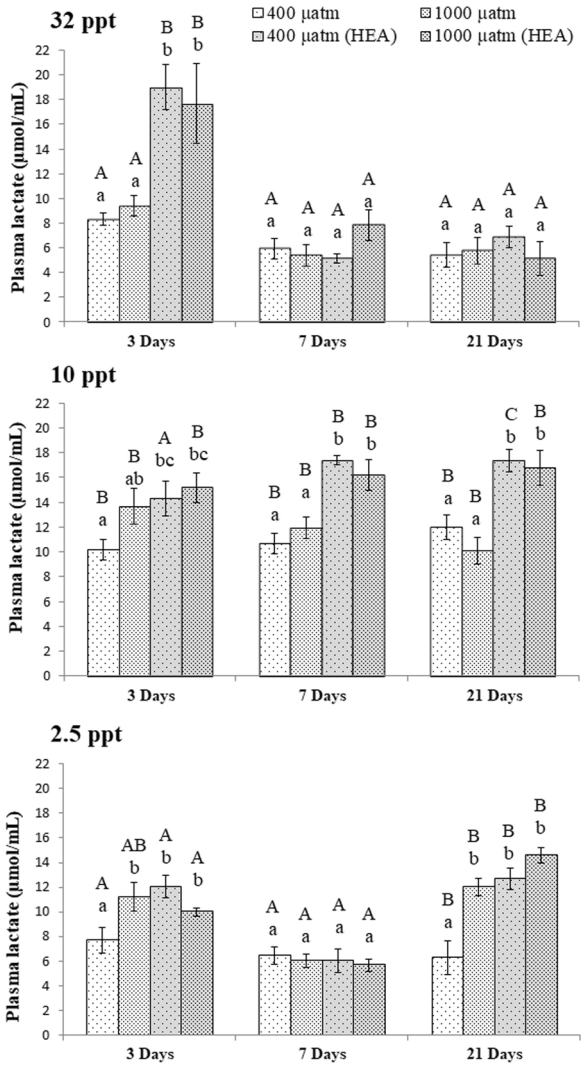

Fig. 5. Lactate accumulation in plasma of fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–c) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

ppt.

3.7. Principal component analysis (PCA)

The PCA biplot depicts a clear separation of experimental groups, mainly along the first two components (PC1 and PC 2) together explaining 56% of data variability (Fig. 12). These differential responses were typically prominent for the hyposaline (2.5 ppt) acclimated fish. The prevailing PC1 component (38% of the data variance) clustered Rhcg isoforms, Rhbg, muscle ammonia content, NKA and NKCC1 expression with the experimental fish groups that were held at 2.5 ppt (with or without $HiCO_2$ exposure, either discrete or in combination with HEA). PC 2 (18% of the data variance) did not reveal a prominent clustering trend.

4. Discussion

To get insight on how fish cope with environmentally relevant stress-mixtures comprising ocean acidification, salinity challenges and elevated ammonia, and to understand their mode of interaction, we evaluated the temporal effects of single and combined exposures of these three waterborne variables on a suite of adaptive responses in European sea bass at various levels of biological organizations.

4.1. Acid-base balance

It has long been understood that OA (elevated pCO_2) can incite a direct effect on acid-base regulation, respiratory function and ionic homeostasis of fish (Cameron and Randall, 1972; Ivanina and Sokolova, 2015). For a number of marine teleosts, elevated water CO_2 resulted in a transient drop of extracellular and intracellular pH values (Larsen et al., 1997; Michaelidis et al., 2007; Perry, 1982; Toews et al., 1983). Ideally, blood pH should be strictly maintained because the reductions in the pH can decrease the affinity of respiratory pigments for oxygen (Bohr effect), and thus adversely affect respiratory function, metabolism as well as aerobic performance of fish. pH compensation in fish can be achieved by direct proton secretion as well as by retention or uptake of HCO_3^- across the gill epithelium. This can eventually lead to the net accumulation of HCO_3^-, as reported for a number of marine teleosts when confronted with elevated CO_2 levels (Deigweiher et al., 2008;

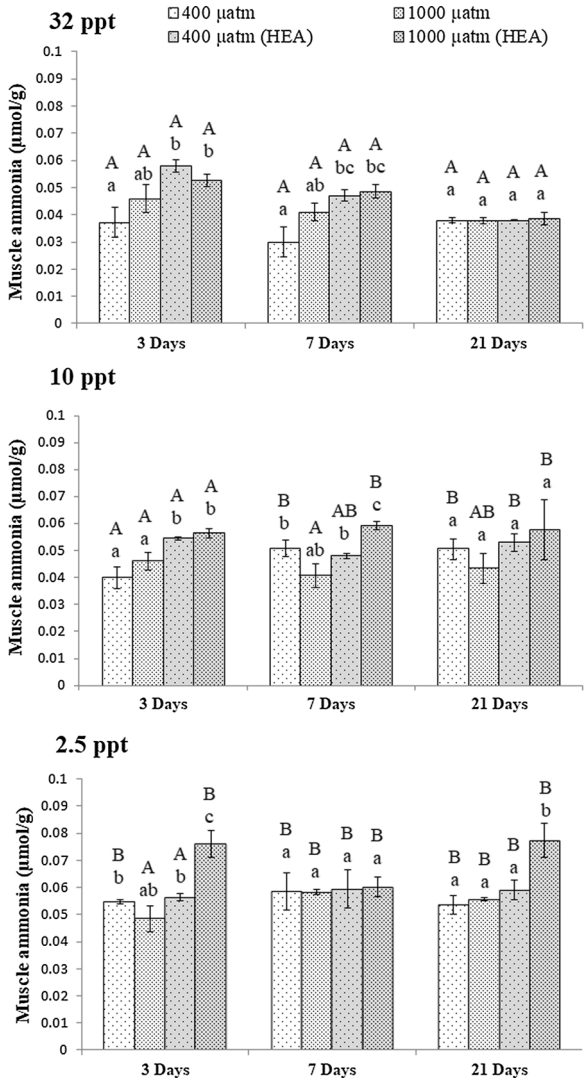

Fig. 6. Muscle ammonia accumulation in fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–c) denotes significant difference between different treatments within same time points. Capital letters (A,B) denotes significant difference between same treatments at different salinities.

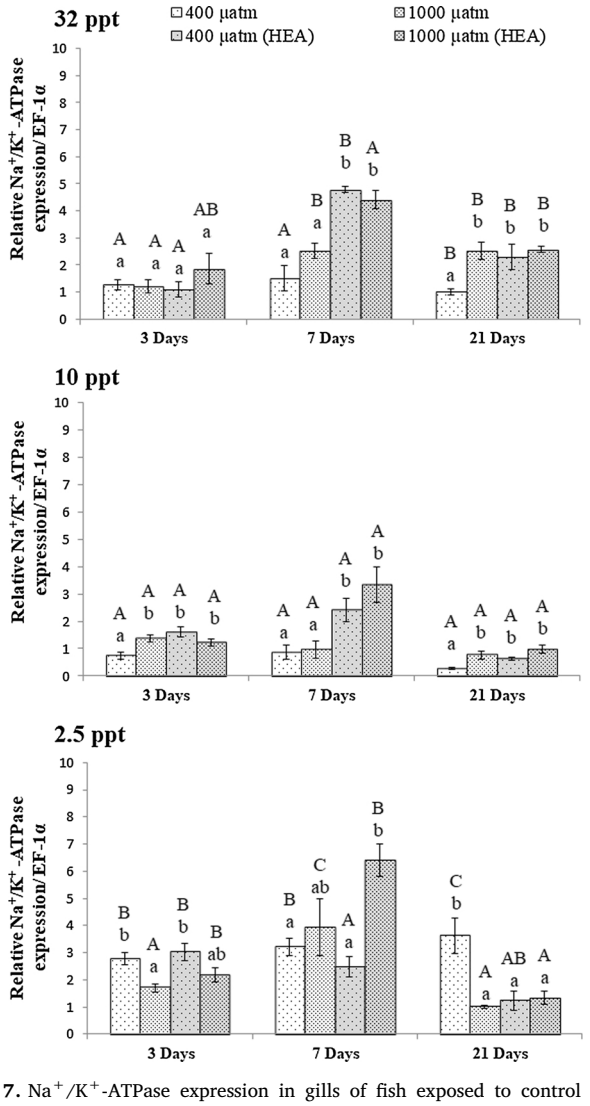

Fig. 7. Na^+/K^+-ATPase expression in gills of fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a,b) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

Heuer and Grosell, 2014; Larsen et al., 1997; Michaelidis et al., 2007). Complete compensation of blood pH acidification to control levels was reported in European seabass (Petochi et al., 2011), Japanese flounder (*Paralichthys olivaceus*) (Hayashi et al., 2004), red drum (*Sciaenops ocellatus*) (Esbaugh et al., 2016) and gulf toadfish (*Opsanus beta*) (Esbaugh et al., 2012) following exposure to elevated CO_2 levels. Likewise, in our study no reduction in blood pH was observed in elevated pCO_2 ($HiCO_2$) groups, suggesting that any blood acidification that may have occurred was already been compensated for, possibly by increased blood HCO_3^- level. We noted that plasma [HCO_3-] and plasma pCO_2 tended to be higher during $HiCO_2$ exposure, but the values were inconsistent with large variations within control groups. This was probably due to the fact that a caudal puncture blood sampling protocol, which has several drawbacks relative to the cannulation, was employed as we used juvenile small-sized fish in the present study. Caudal puncture may result in a mixture of arterial and venous blood, which has different blood gas profiles. In the estuarine red drum, Esbaugh et al. (2016) documented that caudal puncture blood samples had much lower pH and a higher pCO_2 than blood samples collected through cannulation due to the non-steady state CO_2 equilibrium associated with the caudal puncture sampling procedure. However, as stated above, due to the small size of the fish caudal puncture sampling method was the only possibility to get an indication of possible compensation mechanisms.

We found that the degree of blood pH compensation was dependent on the salinity of the environmental water; blood pH under all treatments was notably lower in 2.5 ppt fish than in 32 ppt. This can be ascribed to the low plasma [HCO_3^-] in 2.5 ppt acclimated fish. In addition, it also likely that due to the poorer buffering capacity in lower salinity (2.5 ppt), the compensation for the blood acidosis (via HCO_3-uptake) was weaker compared to 10 ppt and full strength seawater (32 ppt). This is also supported by the work of Iwama and Heisler (1991) in CO_2 exposed rainbow trout (*Oncorhynchus mykiss*) wherein a more efficient acid-base compensation was noted when fish were acclimated to normal seawater compared to freshwater. Furthermore, it should be noted that the pH of the hyposaline water (2.5 ppt) is actually lower than the 10 and 32 ppt water at the same pCO_2 (refer section 2.3.1), which could partially be an explanation for more severe effects in 2.5 ppt -acclimated fish.

At physiological pH (e.g., arterial pH of 7.8) only a small fraction

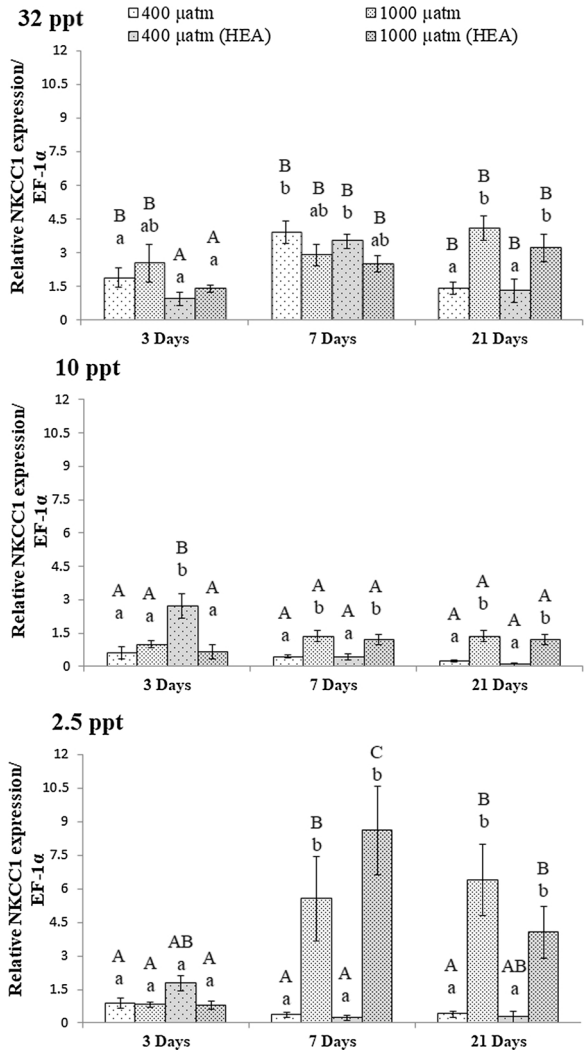

Fig. 8. NKCC1 expression in gills of fish exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a,b) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

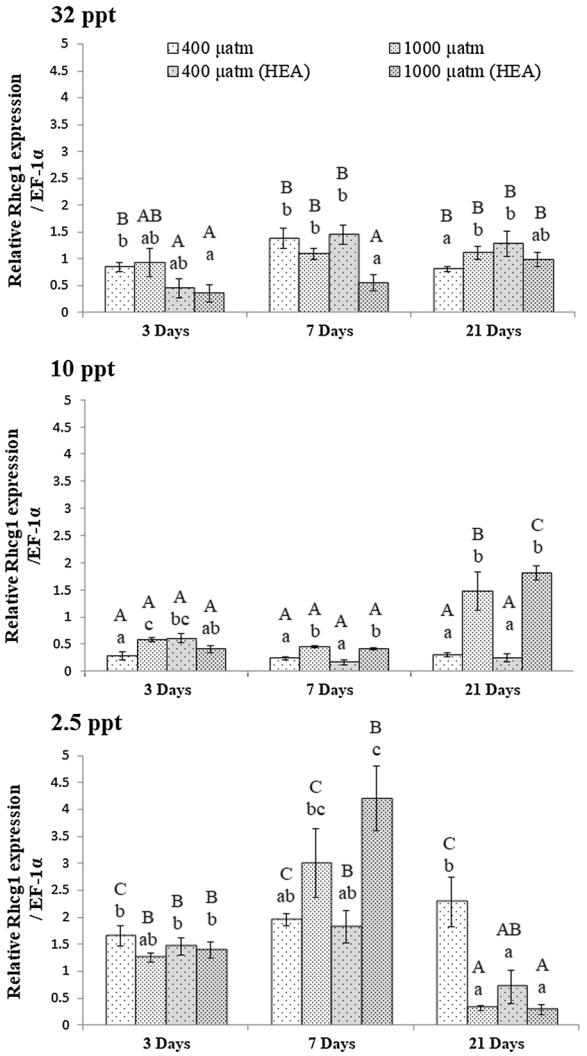

Fig. 9. Expression of Rhcg1 mRNA in the fish gills exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–c) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

(around 5%) of the total ammonia exists as toxic (unionized) NH_3 (Wilkie, 1997, 2002). For each pH unit increase, the amount of unionized toxic ammonia increases by about 10 times (Cameron and Heisler, 1983; Erickson, 1985). In this context, an increment in blood pH in function of HEA exposure in all treatments (pCO_2 and salinity) would lead to a higher level of toxic NH_3 in the blood. Similar to our results, a numerical increment in blood pH was noted in weather loach (*Misgurnus anguillicaudatus*) when exposed to elevated ammonia for 48 h (Tsui et al., 2002). Furthermore, the interplay between the HEA induced blood alkalization on one hand, and possible blood acidosis by $HiCO_2$ exposure on another, may partially explain the dissimilarity in blood pH value among $LoCO_2$-HEA and $HiCO_2$-HEA groups.

Furthermore, blood pH in $HiCO_2$ exposed group reared at 32 ppt and 10 ppt corresponds well with the unchanged level of plasma lactate, further reinforcing that blood acidosis was fully compensated in these salinity regimes. This also signifies that these groups of fish have the ability to cover the ion-osmoregulatory expenses by aerobic metabolism as reflected by the lack of plasma lactate accumulation. However, an induced plasma lactate accumulation in 2.5 ppt-$HiCO_2$ exposed fish highlights the onset of anaerobic metabolism (despite maintaining a normoxic environment), and suggests the mobilization of lactate as substrate for gluconeogenesis to provide fuel for osmotic adaptation during combined exposure to ocean acidification and hypo-osmotic stress. In addition, in the course of HEA exposure in combination with $HiCO_2$, increased anaerobic metabolism in all tested salinities may relate to a higher fuel demand to maintain ion-regulation and for active NH_4^+ excretion. This observation agrees with prior studies showing that high levels of ammonia exposure induced plasma lactate accumulation in fish (Diricx et al., 2013; Sinha et al., 2012, 2015b). However, the exact reason for switching to anaerobic metabolism was not clear; we speculate that European sea bass exposed to HEA at low salinities were experiencing some form of endogenous hypoxia because hypo-osmotic stress as well as elevated ammonia has been shown to disrupt fish gill epithelia (Avella et al., 1993; Cardoso et al., 1996; Lease et al., 2003; Maina, 1990; Sinha et al., 2014; Wilkie, 1997). Regrettably, oxygen consumption rate was not determined in the present study, so we have no direct evidence to support the hypothesis that the fish under these experimental conditions experienced internal hypoxia.

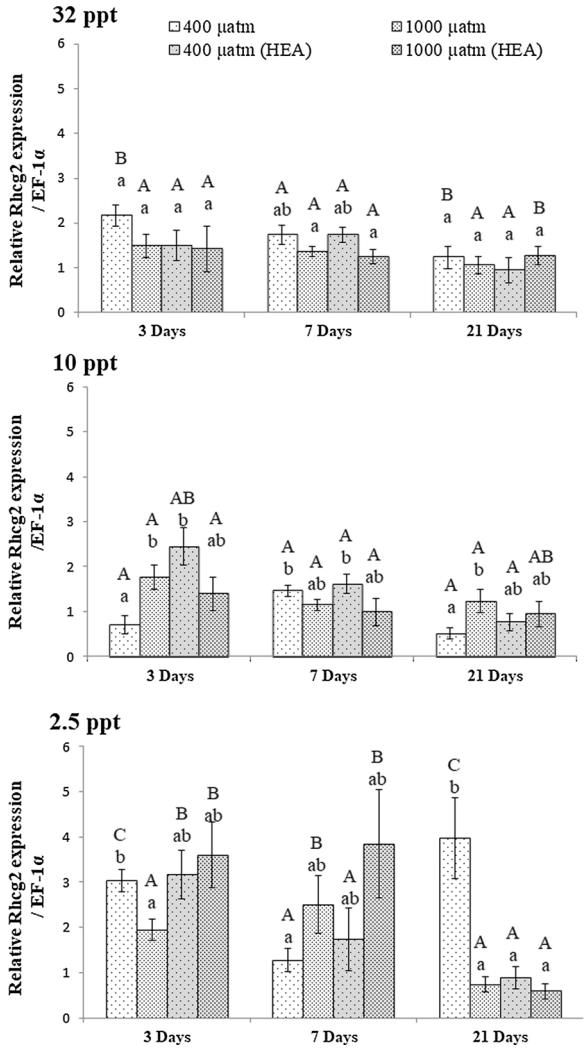

Fig. 10. Expression of Rhcg2 mRNA in the fish gills exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a,b) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

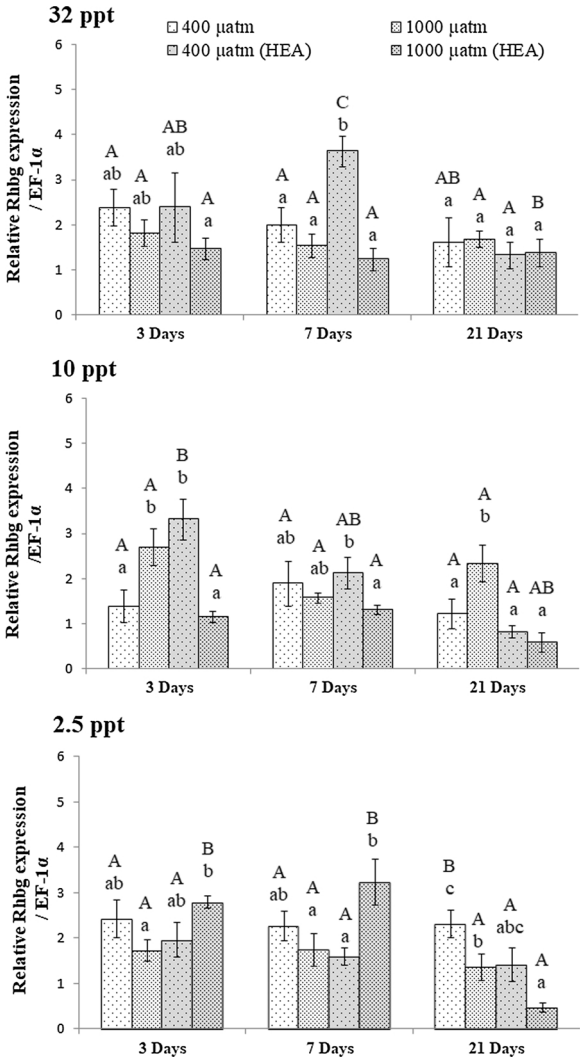

Fig. 11. Expression of Rhbg mRNA in the fish gills exposed to control (400 µatm) and elevated pCO_2 (1000 µatm) conditions during acclimation to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to HEA. Values are mean ± S.E. Different small letters (a–c) denotes significant difference between different treatments within same time points. Capital letters (a–c) denotes significant difference between same treatments at different salinities.

4.2. Implications of Na^+/K^+-ATPase and NKCC for ion-regulation

Na^+/K^+-ATPase (NKA), localized in the basolateral membrane of gill epithelium is widely considered to be the key enzyme providing the driving force for many gradient-dependent transport processes in the gills. Although the response of NKA in fish is studied with regard to salinity stress, osmoregulation, temperature, water borne ammonia and metal toxicity (for reviews, see Evans et al., 2005), till date only a limited amount of studies have investigated its response to OA. The up-regulation in branchial NKA expression to $HiCO_2$ (at seawater and brackish water salinity) corroborates with earlier findings in marine eelpout (*Zoarces viviparous*) that showed significantly higher NKA mRNA level in gill when exposed to 10,000 ppm CO_2 (Deigweiher et al., 2008). Likewise, in red drum and Japanese flounder, the NKA activity was augmented after exposure to 1000 µatm CO_2 and 1%–5% hypercapnia respectively (Esbaugh et al., 2016; Ishimatsu et al., 2005). In Atlantic Cod (*Gadus morhua*), long term acclimation to 0.6 kPa CO_2 displayed a two-fold increased activity of branchial NKA (Melzner et al., 2009). Elevated NKA expression or activity is likely to raise the cytoplasmic Na^+ levels, and thereby increase the Na^+ gradient. As such, this will aid the gradient for HCO_3^- transport via Na^+/HCO_3^- co-transporter (NBC). Therefore, it seems reasonable to speculate that an augmented branchial NKA in response to elevated pCO_2 primarily fuels the need for the increased HCO_3^- uptake (via NBC) as the compensatory response towards blood acidosis. This might also offer another justification for the efficient blood pH compensation documented in the present study following $HiCO_2$ exposure. Nevertheless due to the lack of genomic (sequence) information for NBC in European sea bass, we have no data to support its involvement.

$HiCO_2$ mediated NKA expression was further modulated with the reduction in the osmotic pressure of the rearing medium, which was also clear from the PC1 clusters analysis. We documented that the NKA expression in response to $HiCO_2$ was down-regulated for the fish acclimated at lowest salinity (2.5 ppt). This corroborates with earlier findings in pre-smolts of Atlantic salmon (*Salmo salar*) following elevated CO_2 (20,000 ppm) exposure in freshwater environments (Seidelin et al., 2001). In these cases, lowered NKA functional capacity can have subsiding effects on plasma Na^+ homeostasis (Marshall, 2002; Marshall and Grosell, 2006). Therefore, in order to enhance Na^+ influx in hyposaline or freshwater environments as well as to increase net acid secretion for blood acidosis compensation, the NHE pathway

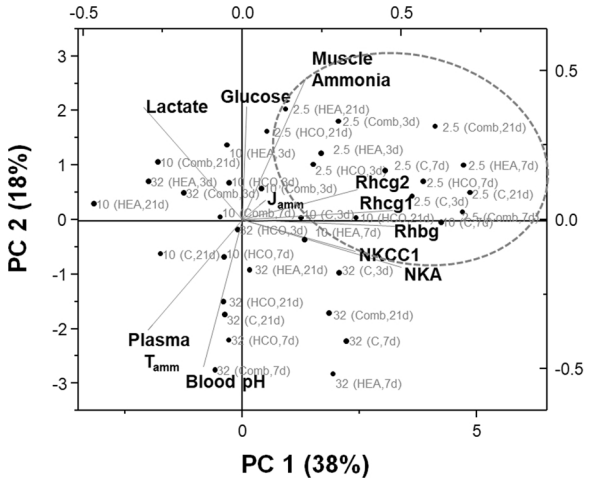

Fig. 12. Principal Component Analysis (PCA) representing the contribution of biochemical parameters for control (400 µatm 'C') and elevated pCO_2 (400 µatm 'HCO') exposure for 3 days (3d), 7 days (7d) and 21 days (21d). The variable coordination is presented by the complementary cases analysis showing distribution of salinity acclimation groups (32, 10, and 2.5 ppt) and high environmental ammonia (HEA) exposure in the (PC 1 × PC 2) coordination plane.

presumably becomes more dominant at lower salinities, especially when challenged simultaneously with ocean acidification (unlike the possible role of NBC in normal seawater and 10 ppt salinity mentioned above). Although not measured in the present study, expression profile of branchial NHE and NBC would offer a precise validation of this hypothesis and is worth further examination.

Gill NKA activity/expression is generally positively correlated with the osmoregulatory ability in marine fish (McCormick, 1995; Seidelin et al., 2001; Sinha et al., 2015a, b), which is also apparent in the present study. Relative to normal seawater (32 ppt) and brackish water (10 ppt), a higher expression level for NKA was recorded for control fish in hyposaline (2.5 ppt) water, possibly as a compensatory mechanism to counteract ion loss induced by the low salinity. These observations also agree with previous studies in European sea bass reared at various lower salinities (Blondeau-Bidet et al., 2019; Sinha et al., 2015a, b). Likewise, branchial NKA capacity were higher in freshwater than in the seawater -acclimated Australian bass (*Maqcuaria novemaculata*) (Langdon, 1987), black seabream (*Mylio macrocephalus*) (Kelly et al., 1999), killifish (*Fundulus heteroclitus*) (Katoh et al., 2002) and milkfish (*Chanos chanos*) (Lin et al., 2003).

NKA is an energy dependent active transport system, involving the hydrolysis of ATP to provide the necessary energy. For its efficient operation, glucose serves as the major metabolic fuel source which is primarily produced by the catabolism of glycogen store (review by Tseng and Hwang, 2008). The elevated expression of branchial NKA in response to $HiCO_2$, HEA and their combination at 32 ppt and 10 ppt was accompanied by an increment in plasma glucose levels, possibly to meet the energy demand required to fuel elevated NKA capacity. However, despite a reduction in NKA expression, glucose level remained elevated in these treatment groups when held at 2.5 ppt. This increased blood glucose might be an adaptive response to fuel instant energy demand by the fish during the adverse hypo-osmotic conditions, and can also be considered as a prime indicator of metabolic response to stresses.

Besides NKA, the contribution of other energy dependent ion-transporters such as H^+-ATPase become important at very low salinities, validated in our previous work on European seabass (Sinha et al., 2015b). H^+-ATPase generates a negative potential inside the apical membrane which drives Na^+ inwards via a sodium channel. As such, at lower salinities the energy is needed to channelize both NKA as well as H^+-ATPase. The fact that glucose level was elevated in 2.5 ppt despite a reduction in NKA expression, suggest an additional fueling of glucose to meet energy demand of H^+-ATPase.

Along with NKA, NKCC is an important ion transport protein, predominantly localized on the basolateral side of the chloride cells in marine teleosts (Lorin-Nebel et al., 2006; Marshall et al., 2002; Wilson et al., 2000). NKCC facilitate Na^+, K^+ and Cl^- transports from blood into the cell (Marshall et al., 2002), and secondarily rely on basolateral NKA to maintain a favorable inward Na^+ gradient by recycling Na^+ back to the extracellular fluid. The intense elevation in branchial NKA transcript noted under various treatments can lead to a decline in the intracellular Na^+ concentration as well as an increased Na^+ gain in the blood. Under these circumstances, an augmentation in transcript level of NKCC1 (parallel or followed by NKA increment) would be a countervailing response to extrude Na^+ load from the blood and maintain intracellular Na^+ homeostasis (Esbaugh et al., 2016; Loong et al., 2012). In addition, a down-regulation of NKCC1 expression (in control groups) at lower salinities (relative to 32 ppt) is possibly a defensive strategy of European sea bass to rapidly control net ion loss. This is in agreement with previous observations in euryhaline fish species when acclimated from seawater to lower salinities (close to freshwater) (Blondeau-Bidet et al., 2019; Hiroi and McCormick, 2007; Katoh et al., 2008; Wilson et al., 2004; Wu et al., 2003). Nevertheless, though there was a fairly good correlation between NKA and NKCC1 at mRNA level, but changes in the gene expression do not always translate into comparable changes in the protein function. Therefore examining the responses of these transporters at the translational level will be crucial in future works.

4.3. Ammonia dynamics

Ammonia is toxic to fish and must be efficiently excreted to avoid build-up in blood and tissue.

In response to $HiCO_2$, ammonia excretion rate (J_{amm}) was either efficiently maintained at basal level (in 32 ppt and 10 ppt) or even elevated (in hyposaline water). This may partly be due to the favorable plasma to water ammonia gradient created by the lowered water pH in $HiCO_2$ groups (as a consequence of the elevated CO_2 hydration in the water, Table 1) and thus create a sink for NH_3 (Claiborne and Heisler, 1986; Larsen and Jensen, 1997; Nawata and Wood, 2008). Lowering of water pH in response to $HiCO_2$ also generate protonation (elevated concentration of extracellular H^+) at the gill/water interface which tends to provide more substitution site for NH_4^+ at the apical $Na^+/H^+(NH_4^+)$ exchanger. In addition, in the hyposaline environment, the necessities to drive the Na^+ influx are in part also facilitated by augmented $Na^+/H^+(NH_4^+)$ exchanger (Evans et al., 2005; Hiroi and McCormick, 2012; Weihrauch et al., 2009). This might further help explaining higher J_{amm} noted for $HiCO_2$-2.5 ppt group. Our results also corroborate earlier findings in rainbow trout that showed significantly higher J_{amm} when exposed to hypercapnia (1% CO_2 in air) in freshwater conditions (Nawata and Wood, 2008). Likewise, environmental hypercapnia has been reported to incite an increase in nitrogen excretion in freshwater teleost common carp (*Cyprinus carpio*) (Claiborne and Heisler, 1986). Alternatively, increased J_{amm} in the $HiCO_2$-2.5 ppt group may also be associated with an increase in internal ammonia production as a result of intensified protein catabolism and deamination. This is also supported from our previous work on European seabass, wherein protein content in liver and muscle tissue in 2.5 ppt acclimated group were relative lower than in their 32 ppt or 10 ppt acclimated counterparts (Sinha et al., 2015b). An efficient ammonia excretion in response to $HiCO_2$ exposure at all salinities was also reflected by relatively stable plasma T_{amm} as well as muscle ammonia levels within the basal level. Also, augmented J_{amm} in $HiCO_2$-2.5 ppt could explain a lower T_{amm} accumulation compared to the $HiCO_2$ exposed fish at 32 ppt and 10 ppt. Exposure to HEA at all tested salinities inhibited J_{amm} at several time points, reflected by a considerable higher plasma T_{amm}, as well as muscle ammonia levels. In addition, J_{amm} was also lowered in $LoCO_2$ fish in function of reduced salinities; this might

offer the justification for considerably higher ammonia accumulation in muscle of 10 ppt and 2.5 ppt acclimated fish relative to those reared at normal seawater salinity. Overall, our findings suggest that ammonia metabolism can be adversely influenced under the synergistic effect of reduced seawater salinity stress and ammonia threat.

In general, ammonia is excreted from fish gills either by diffusion of NH_3 or as the NH_4^+. Gill transporters such as NKA and NKCC are primarily associated with ion transport, but are also believed to be involved in ammonia handling since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999; Wilkie, 1997). NH_4^+ ions are then eliminated to the external medium via the apically localized $Na^+/H^+(NH_4^+)$ antiporter in exchange of Na^+ (Weihrauch et al., 2009; Wilkie, 2002). However, in the present study, NKA or NKCC1 does not seems to link with ammonia efflux pathway, as increased transcript of these transporters in response to exposure conditions ($HiCO_2$ or HEA or combined exposure) did not corresponds with the observed pattern of J_{amm}.

4.4. Significance of Rh glycoproteins as ammonia transporter and CO_2 channels

The majority of ammonia efflux through the gills is believed to be though NH_3 diffusion, either directly or facilitated by Rh glycoproteins (Nakada et al., 2007; Nawata et al., 2007). Though Rh glycoproteins proteins are typically considered NH_3 channels, recent work suggests that these proteins also facilitate CO_2 transport across biological membranes, thereby, possibly act as dual pathways for ammonia and CO_2 transport (Nawata and Wood, 2008; Perry et al., 2010; Tseng et al., 2013). In the gills of European seabass, two Rhcg homologs (Rhcg1 and Rhcg2) were recently identified and are presumably localized in apical membranes (Blondeau-Bidet et al., 2019). Interestingly, the up-regulation of Rhcg1 and Rhcg2 in response to $HiCO_2$ (in seawater and brackish water) corresponds well with the strict compensation of blood acidosis under high CO_2, possibly signifying a potential role of Rh proteins in CO_2 transport as well as acid-base balance regulation in European sea bass under the influence of ocean acidification. Similar to our results, exposure to seawater pCO_2 of 0.12 and 0.42 kPa up-regulated Rhcg gene in gills of medaka (*Oryzias latipes*) (Tseng et al., 2013). Also, a rise in Rhcg1 was reported in the estuarine red drum after 72 h of exposure to ocean acidification condition with 1000 μatm CO_2 level (Esbaugh et al., 2016). Rhbg is believed to be present on the basolateral membranes of the branchial epithelium. Our study shows that Rhbg expression was not induced following $HiCO_2$ challenge (except at 10 ppt). Tseng et al. (2013) however have shown that Rhbg is highly expressed in gills of medaka following exposure to elevated pCO_2, indicating species specific differences.

The hyposaline environment induced a many fold increase in Rhcg1 and Rhcg2 expression relative to normal seawater. PC analysis also confirmed that 2.5 ppt (with or without $HiCO_2$ and HEA exposure) is the prevailing factor clustering the expression dynamics of Rhcg isoforms. This is consistent with the results of Blondeau-Bidet et al. (2019) on European sea bass which displayed a significant increment in Rhcg1 expression when reared in freshwater compared to seawater. Since lower salinity acclimated European sea bass express Rhcg in their gills to a greater extent, higher basal levels of this protein in 2.5 ppt salinity acclimated fish may be sufficient to compensate for their internal acidosis in the course of $HiCO_2$ exposure. As such, any increment in Rhcg in response to $HiCO_2$ at 2.5 ppt (analogous to 32 ppt and 10 ppt) might have been masked by a higher resting expression level. In lower salinities or a freshwater environment, besides the involvement of NKA and other ion-transporters (e.g. NHE, H^+-ATPase), the gradient required for sodium uptake is also linked to Rhcg (Weihrauch et al., 2009). Consequently, it is appealing to speculate that a remarkable upsurge in Rhcg expression in hyposaline (Na^+-poor) medium may be an attempt to empower uptake of Na^+. Likewise, Shih et al. (2008) documented that low-sodium water is a trigger for the activation of Rhcg1 in zebrafish (*Danio rerio*) larvae. No effect of salinity reduction on Rhbg expression agrees with previous observation in freshwater challenged European seabass (Blondeau-Bidet et al., 2019).

A number of reports in freshwater and marine teleosts have shown that high external ammonia can trigger Rh glycoproteins mRNA expression in gills, and thus facilitate their ammonia excretion efficiency (Braun et al., 2009; Egnew et al., 2019; Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2009; Sinha et al., 2013, 2016; Tsui et al., 2009; Zimmer et al., 2010). In present study, Rh glycoprotein also responded to HEA. Among 10 ppt acclimated fish, the strong transcriptional activation of Rhcg isforms and Rhbg in response to HEA at day 3 coincided with the J_{amm} increment within the same time frame. However, in HEA exposed-32 ppt fish, J_{amm} remained inhibited (at day 21) despite a parallel increment in Rhcg1 transcript level. Also, in the same salinity group, neither does the increment in Rhbg expression facilitate J_{amm} under HEA at day 7. The exact reasons for this mis-match between the gene expression and associated phenotypic changes remained unclear. This may be probably due to time-lag effect between transcription and translation and/or post-translational modifications. Furthermore, for the fish group acclimated at the lowest experimental salinity (2.5 ppt), the expressions of Rhcg1, Rhcg2, Rhbg along with NKA were extremely down-regulated following chronic (21 days) $HiCO_2$ exposure either alone or in combination with HEA. This data set indicates that in long term the combined (synergistic) effect of ocean acidification, salinity reduction and ammonia challenge would probably become detrimental and may compromise the fish capacity to maintain acid-base regulation and ammonia homeostasis.

5. Conclusion

Understanding the interactive effects of ocean acidification in combination with other environmental stressors is currently becoming a wide spread concern for climate change researchers. The current study represents the first attempt to mechanistically address the combined effect of elevated pCO_2, reduced seawater salinities and high ammonia on the adaptive capacity of fish. We found that countervailing strategies of European sea bass, a model estuarine teleost, against the adverse effect of elevated pCO_2 and HEA, were differentially modulated under the influence of reduced seawater salinity regimes. Firstly, in response to the individual exposure to $HiCO_2$, the degree of compensation for blood pH was much more efficient at normal seawater (32 ppt) than at lower salinities (10 ppt and 2.5 ppt), which was evident by much lower blood pH value in the latter salinities relative to the former. This seemingly is attributed to a greater HCO_3^- buffering capacity in seawater experimental group. Also, any increment in the blood pH in response to HEA was restored under the combined exposure, indicating that $HiCO_2$ and HEA abolished each other's effect. Secondly, fish exposed to $HiCO_2$ at all salinities was able to maintain J_{amm} within control values of the pCO_2 (400 μatm) group, which was reflected by relatively stable plasma as well as muscle ammonia level. In contrast, for almost all tested salinities, the highest accumulation of ammonia in plasma and muscle tissue was noted for $HiCO_2$-HEA co-exposed groups, concomitant with the reduced J_{amm}. A declining trend of J_{amm} under HEA exposure was also apparent at lowered salinities. Thirdly, unlike in the hyposaline environment (2.5 ppt), an up-regulation of branchial Rhcg1 and Rhcg2 expression in response elevated pCO_2 for 32 ppt and 10 ppt -acclimated fish signifies their potential role as CO_2 channel. However, there was no clear-cut relationship between activated Rh glycoproteins and J_{amm} pattern. Fourthly, analogous to Rh glycoproteins, elevation in NKA transcript under exposure conditions ($HiCO_2$, HEA or both) were seen only in the 32 ppt and 10 ppt groups. These increments are likely an attempt to maintain ionic homeostasis and create gradient for HCO_3^- uptake to avoid respiratory acidosis. An up-regulated NKCC1 transcript level, in parallel or followed by NKA increment, probably aids to maintain intracellular Na^+ homeostasis. Lastly, discrete and co-

exposure of $HiCO_2$ and HEA were found to be potentially stressful as displayed by an increase in plasma glucose and lactate content, however these disturbances occurred relatively earlier in 2.5 ppt groups. In short, the patterns of physiological adaptive strategies, stress response and gene-expression modulation observed in the present study suggest that hyposaline environment as well as HEA sensitizes European seabass juveniles to the negative impacts of global increase in CO_2 level. The changes in the gill transporters were investigated at the mRNA level. In the future investigations, responses at the translational level will be crucial.

Acknowledgments

The technical assistance of Karin Van den Bergh, Steven Joosen, Femke De Croock, Pier (Peter De Feu), Ali Pilehvar and Nemo Maes is gratefully acknowledged. Authors are also thankful to Dr. Raewyn Town for her continual assistance throughout this study. The authors would also like to thank Dr. Amit Kumar Sinha for the critical reading of the manuscript and the helpful suggestions. Jyotsna Shrivastava is a research fellow supported by a PhD Grant [BOF-DOCPRO-31444] from the University of Antwerp Research Council.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2019.04.024.

References

Alam, M., Frankel, T.L., 2006. Gill ATPase activities of silver perch, *Bidyanus bidyanus* (Mitchell), and golden perch, *Macquaria ambigua* (Richardson): effects of environmental salt and ammonia. Aquaculture 251, 118–133.

Allmon, E.B., Esbaugh, A.J., 2017. Carbon dioxide induced plasticity of branchial acid-base pathways in an estuarine teleost. Sci. Rep. 7, 1–10.

Avella, M., Berhaut, J., Bornancin, M., 1993. Salinity tolerance of two tropical fishes, *Oreochromis aureus* and *O. niloticus*. I. Biochemical and morphological changes in the gill epithelium. J. Fish Biol. 42, 243–254.

Baumann, H., Talmage, S.C., Gobler, C.J., 2012. Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat. Clim. Change 2, 38–41.

Blondeau-Bidet, E., Hiroi, J., Lorin-Nebel, C., 2019. Ion uptake pathways in European sea bass *Dicentrarchus labrax*. Gene 692, 126–137.

Braun, M.H., Steele, S.L., Perry, S.F., 2009. The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. J. Exp. Biol. 212, 3846–3856.

Cai, W.J., Hu, X.P., Huang, W.J., Murrell, M.C., Lehrter, J.C., Lohrenz, S.E., Chou, W.C., Zhai, W.D., Hollibaugh, J.T., Wang, Y.C., Zhao, P.S., Guo, X.H., Gundersen, K., Dai, M.H., Gong, G.C., 2011. Acidification of subsurface coastal waters enhanced by eutrophication. Nat. Geosci. 4, 766–770.

Cameron, J.N., Heisler, N., 1983. Studies of ammonia in the rainbow trout: physico-chemical parameters, acid–base behaviour and respiratory clearance. J. Exp. Biol. 105, 07–25.

Cameron, J.N., Randall, D.J., 1972. The effect of increased ambient CO_2 on arterial CO_2 tension, CO_2 content and pH in rainbow trout. J. Exp. Biol. 57, 673–680.

Cardoso, E.L., Chiarini-Garcia, H., Ferreira, R.M.A., Poli, C.R., 1996. Morphological changes in the gills of *Lophiosilurus alexandri* exposed to un-ionized ammonia. J. Fish Biol. 49, 778–787.

Claiborne, J.B., 1998. Acid–base regulation. In: Evans, D.H. (Ed.), The Physiology of Fishes, 2nd ed. pp. 177–198.

Claiborne, J.B., Heisler, N., 1986. Acid–base regulation and ion transfers in the carp (*Cyprinus carpio*) — pH compensation during graded long-term and short-term environmental hypercapnia, and the effect of bicarbonate infusion. J. Exp. Biol. 126, 41–61.

Clements, J.C., Chopin, T., 2016. Ocean acidification and marine aquaculture in North America: potential impacts and mitigation strategies. Rev. Aquac. 1–16.

Cutler, C.P., Cramb, G., 2001. Molecular physiology of osmoregulation in eels and other teleosts: the role of transporter isoforms and gene duplication. Comp. Biochem. Physiol., Part A: Mol. Integr 130, 551–564.

Deigweiher, K., Koschnick, N., Portner, H.O., Lucassen, M., 2008. Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 295, R1660–R1670.

Diricx, M., Sinha, A.K., Liew, H.J., Mauro, N., Blust, R., De Boeck, G., 2013. Compensatory responses in common carp (*Cyprinus carpio*) under ammonia exposure: additional effects of feeding and exercise. Aquat. Toxicol. 142, 123–137.

Dore, J.E., Lukas, R., Sadler, D.W., Church, M.J., Karl, D.M., 2009. Physical and biogeochemical modulation of ocean acidification in the central North Pacific. Proc. Natl. Acad. Sci. 106, 12235–12240.

Egnew, N., Renukdas, N., Ramena, Y., Yadav, A.K., Kelly, A.M., Lochmann, R.T., Sinha, A.K., 2019. Physiological insights into largemouth bass (*Micropterus salmoides*) survival during long-term exposure to high environmental ammonia. Aquat. Toxicol. 207, 72–82.

Ellis, R.P., Urbina, M.A., Wilson, R.W., 2017. Lessons from two high CO_2 worlds–future oceans and intensive aquaculture. Glob. Change Biol. Bioenergy 23, 2141–2148.

Erickson, R.J., 1985. An evaluation of mathematical models for the effects of pH and temperature on ammonia toxicology to aquatic organisms. Water Res. 19, 1047–1058.

Esbaugh, A.J., Heuer, R., Grosell, M., 2012. Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. J. Comp. Physiol. B. 182, 921–934.

Esbaugh, A.J., Ern, R., Nordi, W.M., Johnson, A.S., 2016. Respiratory plasticity is insufficient to alleviate blood acid–base disturbances after acclimation to ocean acidification in the estuarine red drum, Sciaenops ocellatus. J. Comp. Physiol. B. 186, 97–109.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

FAO, 2012. The State of the World Fisheries and Aquaculture. Food and Agriculture Organisation of the United Nations, Rome.

FAO, 2014. The State of World Fisheries and Aquaculture - Opportunities and Challenges. Food and Agriculture Organization of the United Nations, Rome.

Feely, R.A., Alin, S.R., Newton, J., Sabine, C.L., Warner, M., Devol, A., Krembs, C., Maloy, C., 2010. The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. Estuar. Coast. Shelf Sci. 88, 442–449.

Geay, F., Santigosa, I., Culi, E., Corporeau, C., Boudry, P., Dreano, Y., Corcos, L., Bodin, N., Vandeputte, M., Zambonino-Infante, J.L., Mazurais, D., Cahu, C.L., 2010. Regulation of FADS2 expression and activity in European sea bass (*Dicentrarchus labrax*: L.) fed a vegetable diet. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 156, 237–243.

Giffard-Mena, I., Lorin-Nebel, C., Charmantier, G., Castille, R., Boulo, V., 2008. Adaptation of the sea bass (*Dicentrarchus labrax*) to fresh water: role of aquaporins and Na^+/K^+-ATPases. Comp. Biochem. Physiol., Part A: Mol. Integr. 150, 332–338.

Green, L., Jutfelt, F., 2014. Elevated carbon dioxide alters the plasma composition and behaviour of a shark. Biol. Lett. 10, 20140538.

Hakim, Y., Harpaz, S., Uni, Z., 2009. Expression of brush border enzymes and transporters in the intestine of European sea bass (*Dicentrarchus labrax*) following food deprivation. Aquaculture 290, 110–115.

Hayashi, M., Kita, J., Ishimatsu, A., 2004. Acid–base responses to lethal aquatic hypercapnia in three marine fishes. Mar. Biol. 144, 153–160.

Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 307, R1061–R1084.

Heuer, R.M., Welch, M.J., Rummer, J.L., Munday, P.L., Grosell, M., 2016. Altered brain ion gradients following compensation for elevated CO_2 are linked to behavioural alterations in a coral reef fish. Sci. Rep. 6, 33216.

Hiroi, J., McCormick, S.D., 2007. Variation in salinity tolerance, gill Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. J. Exp. Biol. 210, 1015–1024.

Hiroi, J., McCormick, S.D., 2012. New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. Respir. Physiol. Neurobiol. 184, 257–268.

Hirose, S., Kaneko, T., Naito, N., Takei, Y., 2003. Molecular biology of major components of chloride cells. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 136, 593–620.

Hung, C.Y.C., Tsui, K.N.T., Wilson, J.M., Nawata, C.M., Wood, C.M., Wright, P.A., 2007. Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J. Exp. Biol. 210, 2419–2429.

Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion rich cells. Comp. Biochem. Physiol., Part A: Mol. Integr. 148, 479–497.

Hwang, P.P., Lee, T.H., Lin, L.Y., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. J. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, 28–47.

IPCC, 2013. Climate Change: The Physical Science Basis Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. p. 2013.

IPCC, 2014. Climate change: impacts, adaptation, and vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press 2014.

Ishimatsu, A., Hayashi, M., Lee, K.S., 2005. Physiological effects on fishes in a high-CO_2 world. J. Geophys. Res. 110, C09S09.

Ivanina, A.V., Sokolova, I.M., 2015. Interactive effects of metal pollution and ocean acidification on physiology of marine organisms. Current Zool. 61, 653–668.

Iwama, G.K., Heisler, N., 1991. Effect of environmental water salinity on acid-base regulation during environmental hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 158, 1–18.

Joos, F., Spahni, R., 2008. Rates of change in natural and anthropogenic radiative forcing over the past 20,000 years. Proc. Natl. Acad. Sci. 105, 1425–1430.

Katoh, F., Shimizu, A., Uchida, K., Kaneko, T., 2002. Shift of chloride cell distribution during early life stages in seawater adapted killifish Fundulus heteroclitus. Zool. Sci. 17, 11–18.

Katoh, F., Cozzi, R.R.F., Marshall, W.S., Goss, G.G., 2008. Distinct $Na^+/K^+/2Cl^-$ co-transporter localization in kidneys and gills of two euryhaline species: rainbow trout

and killi fish. Cell Tissue Res. 334, 265–281.
Kelly, S.P., Woo, N.Y.S., 1999. The response of sea bream following abrupt hyposmotic exposure. J. Fish Biol. 55, 732–750.
Kelly, S.P., Chow, I.N.K., Woo, N.Y.S., 1999. Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. J. Exp. Zool. 283, 226–241.
Langdon, J.S., 1987. Active osmoregulation in the Australian bass, *Macquaria novemaculata* (Steindachner), and the golden perch: *macquaria ambigua* (Richardson) (Percichthyidae). Aust. J. Mar. Freshwater Res. 38, 771–776.
Larsen, B.K., Jensen, F.B., 1997. Influence of ionic composition on acid-base regulation in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental hypercapnia. Fish Physiol. Biochem. 16, 157–170.
Larsen, B.K., Pörtner, H.O., Jensen, F.B., 1997. Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. Mar. Biol. 128, 337–346.
Lease, H.M., Hansen, J.A., Bergman, H.L., Meyer, J.S., 2003. Structural changes in gills of Lost River suckers exposed to elevated pH and ammonia concentrations. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 134, 491–500.
Lin, Y.M., Chen, C.N., Lee, T.H., 2003. The expression of gill Na K-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. Comp. Biochem. Physiol., Part A: Mol. Integr. 135, 489–497.
Lin, C.H., Huang, C.L., Yang, C.H., Lee, T.H., Hwang, P.P., 2004a. Time-course changes in the expression of Na^+, K^+-ATPase and the morphometry of mitochondrion-rich cells in gills of euryhaline tilapia (*Oreochromis mossambicus*) during freshwater acclimation. J. Exp. Zool. A Comp. Exp. Biol. 301, 85–96.
Lin, C.H., Tsai, R.S., Lee, T.H., 2004b. Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp. Biochem. Physiol., Part A: Mol. Integr. 138, 287–295.
Loong, A.M., Chew, S.F., Wong, W.P., Lam, S.H., Ip, Y.K., 2012. Both seawater acclimation and environmental ammonia exposure lead to increases in mRNA expression and protein abundance of Na^+:K^+:$2Cl^-$ cotransporter in the gills of the freshwater climbing perch, *Anabas testudineus*. J. Comp. Physiol. B. 182, 491–506.
Lorin-Nebel, C., Boulo, V., Bodinier, C., Charmantier, G., 2006. The $Na^+/K^+/2Cl^-$ co-transporter in the sea bass *Dicentrarchus labrax* during ontogeny: involvement in osmoregulation. J. Exp. Biol. 209, 4908–4922.
Maina, J.N., 1990. A study of the morphology of the gills of an extreme alkalinity and hyperosmotic adapted teleost *Oreochromis alcalicus grahami* (Boulenger) with particular emphasis on the ultrastructure of the chloride cells and their modifications with water dilution. Anat. Embryol. 181, 83–98.
Marshall, W.S., 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. J. Exp. Zool. 293, 264–283.
Marshall, W.S., Bryson, S.E., 1998. Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp. Biochem. Physiol., Part A: Mol. Integr. 119, 97–106.
Marshall, W.S., Grosell, M., 2006. Ion transport, osmoregulation, and acid-base balance. In: Evans, D.H., Claiborne, J.B. (Eds.), The Physiology of Fishes, 3rd edn. Taylor and Francis Group, New York, pp. 177–230.
Marshall, W.S., Lynch, E.M., Cozzi, R.R.F., 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter inchloride cells during adaptation to the killifish *Fundulus heteroclitus* to seawater. J. Exp. Biol. 205, 1265–1273.
McCormick, S.D., 1995. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: In: Wood, C.M., Shuttleworth, T.J. (Eds.), Fish Physiology, Ionoregulation: Cellular and Molecular Approaches, vol. XIV. Academic Press, New York, pp. 285–315.
Melzner, F., Göbel, S., Langenbuch, M., Gutowska, M.A., Pörtner, H.O., Lucassen, M., 2009. Swimming performance in Atlantic Cod (*Gadus morhua*) following long-term (4–12 months) acclimation to elevated seawater PCO_2. Aquat. Toxicol. 92, 30–37.
Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M.A., Bange, H.W., Hansen, H.P., Kortzinger, A., 2013. Future ocean acidification will be amplified by hypoxia in coastal habitats. Mar. Biol. 160, 1875–1888.
Meure, C.M., Etheridge, D., Trudinger, C., Steele, P., Langenfelds, R., van Ommen, T., Smith, A., Elkins, J., 2006. Law dome CO_2, CH_4 and N_2O ice core records extended to 2000 years BP. Geophys. Res. Lett. 33, L14810.
Michaelidis, B., Spring, A., Pörtner, H.O., 2007. Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish Sparus aurata. Mar. Biol. 150, 1417–1429.
Miller, G.M., Watson, S., Donelson, J.M., McCormick, M.I., Munday, P.L., 2012. Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nat. Clim. Change 2, 858–861.
Nakada, T., Westhoff, C.M., Kato, A., Hirose, S., 2007. Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J. 21, 1067–1074.
Nawata, C.M., Wood, C.M., 2008. The effects of CO2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. J. Exp. Biol. 211, 3226–3236.
Nawata, C.M., Wood, C.M., 2009. mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout. J. Comp. Physiol. B. 179, 799–810.
Nawata, C.M., Hung, C.C.Y., Tsui, T.K.N., Wilson, J.M., Wright, P.A., Wood, C.M., 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol. Genomics 31, 463–474.
Nawata, C.M., Hirose, S., Nakada, T., Wood, C.M., Kato, A., 2010. Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. J. Exp. Biol. 213 (3150), 3160.
Perry, S.F., 1982. The regulation of hypercapnic acidosis in two salmonids, the freshwater trout (*Salmo gairdneri*) and the seawater salmon (*Onchorynchus kisutch*). Mar. Behav. Physiol. 9, 73–79.
Perry, S.F., Braun, M.H., Noland, M., Dawdy, J., Walsh, P.J., 2010. Do zebrafish Rh proteins act as dual ammonia-CO_2 channels? J. Exp. Zool. A. Ecol. Genet. Physiol. 313, 618–621.
Person-Le Ruyet, J., Chartois, H., Quemener, L., 1995. Comparative acute ammonia-toxicity in marine fish and plasma ammonia response. Aquaculture 136, 181–194.
Petochi, T., Di Marco, P., Priori, A., Finoia, M.G., Mercatali, I., Marino, G., 2011. Coping strategy and stress response of European sea bass *Dicentrarchus labrax* to acute and chronic environmental hypercapnia under hyperoxic conditions. Aquaculture 315, 312–320.
Pierrot, D., Lewis, E., Wallace, D.W.R., 2006. MS excel program developed for CO_2system calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center. Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee.
Randall, D.J., Wilson, J.M., Peng, K.W., Kok, T.W.K., Kuah, S.S.L., Chew, S.F., Lam, T.J., Ip, Y.K., 1999. The mudskipper *Periophthalmodon schlosseri*, actively transports NH_4^+ against a concentration gradient. Am. J. Physiol. Regul. Integr. Comp.Physiol. 277, 1562–1567.
Roessig, J.M., Woodley, C.M., Cech, J.J., Hansen, L.J., 2004. Effects of global climate change on marine and estuarine fishes and fisheries. Rev. Fish Biol. Fish. 14, 251–275.
Russell, J.M., 2000. Sodium-potassium-chloride cotransport. Physiol. Rev. 80, 211–276.
Seidelin, M., Brauner, C.J., Jensen, F.B., Madsen, S.S., 2001. Vacuolar-type H^+-ATPase and Na^+, K^+-ATPase expression in gills of Atlantic salmon (*Salmo salar*) during isolated and combined exposure to hyperoxia and hypercapnia in fresh water. Zool. Sci. 18, 1199–1205.
Shih, T.H., Horng, J.L., Hwang, P.P., Lin, L.Y., 2008. Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. Am. J. Physiol., Cell Physiol. 295, C1625–1632.
Sinha, A.K., Liew, H.J., Diricx, M., Blust, R., De Boeck, G., 2012. The interactive effects of ammonia exposure: nutritional status and exercise on metabolic and physiological responses in goldfish (*Carassius auratus* L.). Aquat. Toxicol. 109, 33–46.
Sinha, A.K., Liew, H.J., Nawata, C.M., Blust, R., Wood, C.M., De Boeck, G., 2013. Modulation of Rh glycoproteins, ammonia excretion and Na^+ fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia. J. Exp. Biol. 216, 2917–2930.
Sinha, A.K., Matey, V., Giblen, T., Blust, R., De Boeck, G., 2014. Gill remodeling in three freshwater teleosts in response to high environmental ammonia. Aquat. Toxicol. 155, 166–180.
Sinha, A.K., Dasan, A.F., Rasoloniriana, R., Pipralia, N., Blust, R., De Boeck, G., 2015a. Hypoosmotic stress induced physiological and ion-osmoregulatory responsesin European sea bass (*Dicentrarchus labrax*) are modulated differentially by nutritional status. Comp. Biochem. Physiol., Part A: Mol. Integr. 181, 87–99.
Sinha, A.K., Rasoloniriana, R., Dasan, A.F., Pipralia, N., Blust, R., De Boeck, G., 2015b. Interactive effect of high environmental ammonia and nutritional status on ecophysiological performance of European sea bass (*Dicentrarchus labrax*) acclimated to reduced seawater salinities. Aquat. Toxicol. 160, 39–56.
Sinha, A.K., Kapotwe, M., Dabi, S.B., da Silva Montes, C., Shrivastava, J., Blust, R., De Boeck, G., 2016. Differential modulation of ammonia excretion, Rhesus glycoproteins and ion-regulation in common carp (*Cyprinus carpio*) following individual and combined exposure to waterborne copper and ammonia. Aquat. Toxicol. 170, 129–141.
Strobel, A., Bennecke, S., Leo, E., Mintenbeck, K., Portner, H.O., Mark, F.C., 2012. Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. Front. Zool. 9, 28.
Toews, D.P., Holeton, G.F., Heisler, N., 1983. Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. J. Exp. Biol. 107, 9–20.
Tresguerres, M., Hamilton, T.J., 2017. Acid–base physiology, neurobiology and behaviour in relation to CO2-induced ocean acidification. J. Exp. Biol. 220, 2136–2148.
Tseng, Y.C., Hwang, P.P., 2008. Some insights into energy metabolism for osmoregulation in fish. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 148, 419–429.
Tseng, Y.C., Hu, M.Y., Stumpp, M., Lin, L.Y., Melzner, F., Hwang, P.P., 2013. CO_2-driven seawater acidification differentially affects development and molecular plasticity along life history of fish (*Oryzias latipes*). Comp. Biochem. Physiol., Part A: Mol. Integr. 165, 119–130.
Tsui, T.K.N., Randall, D.J., Chew, S.F., Jin, Y., Wilson, J.M., Ip, Y.K., 2002. Accumulation of ammonia in the body and NH_3 volatilization from alkaline regions of the body surface during ammonia loading and exposure to air in the weather loach *Misgurnus anguillicaudatus*. J. Exp. Biol. 205, 651–659.
Tsui, T.K.N., Hung, C.Y.C., Nawata, C.M., Wilson, J.M., Wright, P.A., Wood, C.M., 2009. Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. J. Exp. Biol. 212, 878–892.
Verdouw, H., Van Echteld, C.J.A., Dekkers, E.M.J., 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Res. 12, 399–402.
Wallace, R.B., Baumann, H., Grear, J.S., Aller, R.C., Gobler, C.J., 2014. Coastal ocean acidification: the other eutrophication problem. Estuar. Coast. Shelf Sci. 148, 1–13.
Wedemeyer, G.A., 1997. Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture, Society for Experimental Biology Seminar Series, 62. Cambridge University Press, Cambridge, U.K, pp. 35–71.
Weihrauch, D., Wilkie, M.P., Walsh, P.J., 2009. Ammonia and urea transporters in gills of fish and aquatic crustaceans. J. Exp. Biol. 212, 1716–1730.
Wilkie, M.P., 1997. Mechanisms of ammonia excretion across fish gills. Comp. Biochem. Physiol., Part A: Mol. Integr. 118, 39–50.
Wilkie, M.P., 2002. Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J. Exp. Zool. 293, 284–301.
Wilson, J.M., Randall, D.J., Donowitz, M., Vogl, A.W., Ip, Y.K., 2000. Immunolocalization of ion-transport proteins to branchial epitheliummitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). J. Exp. Biol. 203, 2297–2310.
Wilson, J.M., Antunes, J.C., Bouça, P.D., Coimbra, J., 2004. Osmoregulatory plasticity of

the glass eel of *Anguilla anguilla*: freshwater entry and changes in branchial ion-transport protein expression. Can. J. Fish. Aquat. Sci. 61, 432–442.
Wright, P.A., Felskie, A., Anderson, P.M., 1995. Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. J. Exp. Biol. 198, 127–135.
Wu, Y.C., Lin, L.Y., Lee, T.H., 2003. Na^+, K^+, $2Cl^-$ cotransporter: a novel marker for identifying freshwater- and seawater-type mitochondria- rich cells in gills of the euryhaline tilapia *Oreochromis mossambicus*. Zool. Stud. 42, 186–192.
Yan, M., Li, Z., Xiong, B., Zhu, J., 2004. Effects of salinity on food intake, growth, and survival of pufferfish (*Fugu obscurus*). J. Appl. Ichthyol. 20, 146–149.
Zimmer, A.M., Nawata, C.M., Wood, C.M., 2010. Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. J. Comp. Physiol. B 180, 1191–1204.